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(54) Title: LIPOSOMAL CAMPTOTHECIN FORMULATIONS

(57) Abstract

Liposomal encapsulated camptothecin formulations are provided. The liposomes have improved pharmacokinetics, enhanced efficacy as anti–tumor agents and provide an increased therapeutic index as compared to the free drug and topotecan. The formulations include liposomes comprising at least one phospholipid and a camptothecin or analog thereof.

LIPOSOMAL CAMPTOTHECIN FORMULATIONS

Field of the Invention

5   This invention relates to the fields of biochemistry and medicine, and in particular to

novel liposomal formulations and process for making such formulations. More specifically,

this invention relates to liposomal formulations containing camptothecin and analogs thereof.

Further, this invention relates to methods of manufacturing and of using such formulations.

10   Background of the Invention

Camptothecin is a pentacyclic plant alkaloid originally isolated from the bark of

Camptotheca accuminata trees indigenous to China (Wall *et al.*, J. Am. Chem. Soc., 94:

388 (1966)). The drug contains a fused ring structure incorporating quinoline, pyrrolidine,

alpha-pyridone and a six membered lactone ring. The naturally occurring form of

15   camptothecin is optically active, with the asymmetric carbon atom at position 20 of the

lactone ring in the "S" configuration. Camptothecin and numerous analogs thereof

(hereafter termed camptothecins) are currently the focus of intensive study due to the

potent anti-tumor activity displayed by these compounds both *in vitro* and *in vivo* (e.g.,

Giovanella, *et al.*, Science 246: 1046-1048 (1989)). The cytotoxic effects of

20   camptothecins have also been exploited in their use as anti-viral, anti-Plasmodium and

anti-haemoflagellate agents (Priel *et al.*, U.S. Patent No. 5,622,959; Priel *et al.*, U.S. Patent

No. 5,422,344; Atlas, WO 9611005; Wall *et al.*, U.S. Patent No. 5,614,529; Shapiro *et al.*,

U.S. Patent No. 5,496,830; Pardee, WO 9404160).

Camptothecin drugs are believed to exert their anti-tumor effect by binding to and

25   reversibly inhibiting the action of the enzyme topoisomerase I. This enzyme is required

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for DNA and RNA synthesis in proliferating cells, where it catalyses the relaxation of

supercoiled DNA structures that form during these processes. As a result of

topoisomerase I inhibition, the biosynthesis of nucleic acids is strongly inhibited leading to

DNA breakage and cell death.

5   Despite their impressive antitumor properties, the use of camptothecins includes a

number of drawbacks. First, some camptothecins are extremely insoluble in aqueous

solution, making parenteral administration of the drug problematic. Second, the lactone

ring is susceptible to hydrolysis at the pH of blood plasma, resulting in a carboxylate form

of the drug that has significantly reduced topoisomerase activity (Fassberg and Stella

10   (1992) J. Pharm. Sci. 81(7):676-689; Mi et al. (1995) Biochemistry 34(42):13722-13728;

Potmesil (1994) Cancer Res. 54:1431-1439; Slichenmyer et al. (1993) J. Natl. Cancer Inst.

85:271-291; Jaxel et al. (1989) Cancer Res. 49:5077-5082). Moreover, the carboxylate

form is highly toxic, often inducing gastrointestinal toxicity, myelosuppression and

hemorrhagic cystitis. The biologically active lactone form is also toxic.

15   As a result of these limitations, much effort has been invested in the synthesis of

semi-synthetic camptothecin derivatives with increased water solubility, decreased toxicity

and increased resistance to hydrolysis. Two examples are topotecan (Hycamtin ™)

(*Kingsbury et al.*, J. Med. Chem. 34:98 (1991); Boehm *et al.*, European Patent Application

No. 321,122) which is approved for salvage therapy of metastatic ovarian carcinoma, and

20   irinotecan (Miysaka *et al.*, U.S. Patent No. 4,604,463) which is approved for salvage

therapy of colon cancer. Other derivatives of camptothecin and anti-tumor treatments

using these derivatives are described in Wall *et al.* (U.S. Patent No. 5,340,817); Wall *et al.*

(U.S. Patent No. 5,364,858); Wall *et al.* (U.S. Patent No. 5,244,903); Wall (U.S. Patent

No. 5,180,722); Wall *et al.* (U.S. Patent No. 5,227,380); Wall *et al.* (U.S. Patent No.

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5,049,668); Wani *et al.* (U.S. Patent No. 5,122,606); Giovanella *et al.* (U.S. Patent No.

5,225,404); Giovanella *et al.* (U.S. Patent No. 5,552,154); Wall *et al.* (U.S. Patent No.

5,401,707); Wall *et al.* (U.S. Patent No. 5,122,526); Johnson *et al.* (WO 9311770);

Johnson *et al.* (WO 9214471); Johnson *et al.* (WO 9214469); and Cherian (WO 9611669).

5   Recently, Luzzio *et al.* (U.S. Patent No. 5,559,235) disclosed a camptothecin

derivative that is moderately soluble in aqueous media, while retaining topoisomerase I

activity *in vitro* and anti-tumor activity *in vivo*. This compound, 7-(4-

methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin dihydrochloride,

(also known as GI147211C or lurtotecan) is less toxic overall than camptothecin, and is 5-

10   10 times more potent than topotecan in tumor cell cytotoxicity assays (Emerson *et al.*

(1995) Cancer Research 55:603-609). Some of the stability samples from the clinical trial

formulations of GI147211C were reported to contain a sulfate-GI147211C precipitate as a

result of residual levels of sulfate in glass vials (Tong et al. (1996) PDA Journal of

Pharmaceutical Science and Technology 50(5):326-329). As a practical and efficient

15   therapeutic it would be desirable to administer this compound in a manner that provides an

improved therapeutic index.

       Insoluble, hydrolyzable compounds may be administered in a clinical situation by

packaging the compounds into lipid aggregates or constructs such as liposomes or

micelles. Liposomes are known to be physiologically compatible and biodegradable

20   delivery systems for a broad range of drugs. Furthermore, as solvation in aqueous media

is not required, an insoluble compound can be delivered to the site of action in a more

concentrated and easily administered medicament than the free drug alone.

       Burke (U.S. Patent No. 5,552,156) discloses liposome-associated camptothecins in

which it is postulated that the lactone ring of the camptothecin intercalates with the acyl

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chains of the lipid bilayer. The lactone ring is effectively removed from the aqueous

environment in the interior of the liposome, and protected from hydrolysis. Burke also

describes how the pH of the internal liposome compartment can be lowered so that

camptothecin derivatives with lower affinity for the liposome membranes can reside

5   within the aqueous interior without undergoing hydrolysis. *In vivo* tests demonstrating the

stability of the formulations were not performed.

Constantinides *et al.* (WO 95/08986) discloses formulations of camptothecin and

its structurally related analogs in liposomes having at least 80% of the drug incorporated in

the lipid bilayer. Pharmacokinetic studies were performed on an egg phosphatidylcholine,

10   egg phosphatidylglycerol, camptothecin formulation. The liposomes were multilamellar

and the camptothecin was passively entrapped. Increased plasma levels of camptothecin

were observed over a 4-hour period after administration of the liposomal formulation as

compared to those obtained with the free drug. A larger area under the plasma

concentration-time curve (AUC) was obtained from liposomal versus free camptothecin,

15   although the increase of the AUC was only about 4 fold higher.

Camptothecins incorporated within vesicles and liposomes are also described in

Slater *et al.* (WO 9426253) and Castor *et al.* (WO 9615774).

Summary of the Invention

20   The present invention provides for liposomal formulations of camptothecin and its

structurally related analogs as well as methods for their preparation. The liposomes have

improved pharmacokinetics, enhanced efficacy as anti-tumor agents, and provide an

improved therapeutic index as compared to the free drug. The formulations include

liposomes comprising at least one phospholipid and a camptothecin or analog thereof

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(referred to collectively herein as "camptothecin"). In one embodiment, the formulations

include liposomes comprised of cholesterol, a phosphatidylcholine, an excipient, wherein the

excipient is sulfate or citrate, and a camptothecin, wherein a portion of the camptothecin may

be precipitated in the aqueous interior of the liposomes in the presence of the excipient. The

5   preferred camptothecin for use in this invention is GI147211. Further, the formulations

described herein are stable upon storage.

Brief Description of the Figures

        Figure 1 depicts the precipitation of GI147211 with various counterion excipients.

10   **Figure 2** depicts the stability in two representative liposomal GI147211 lots prepared

for comparative efficacy.

        Figures 3A-3D show liposomal GI147211 cytotoxicity vs. GI147211 and

topotecan (TP). A) ES-2 (ovarian carcinoma), B) KB (oralpharyngial carcinoma), C)

SKOV-3 (ovarian carcinoma), and D) KBV (oralpharyngial carcinoma, vincristine

15   resistant phenotype) tumor cell proliferation by GI147211C, liposomal GI147211 and TP

described by ^{3}H-Thymidine Incorporation (CPM) vs. Concentration [ng/ml] values. IC_{50}

values (ng/ml) calculated for each data set are presented below each panel graph. ■ =

GI147211C, ◆ = liposomal GI147211, and ▼ = TP.

        Figure 4A-4D show liposomal GI147211 cytotoxicity v. GI147211 and empty

20   liposome A) A549 (lung carcinoma), B) DU-145 (prostate carcinoma), C) KB

(oralpharyngial carcinoma), and D) LOX (melanoma) tumor cell proliferation by

GI147211C, liposomal GI147211 and empty liposomes described by ^{3}H-Thymidine

Incorporation (CPM) vs. Concentration [ng/ml] values. IC_{50} values (ng/ml) calculated for

each data set are presented below each panel graph. All tumor cell types are of human

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derivation. ■ = GI147211C, ▲ = liposomal GI147211 (Lot # ALM 993-028), and ▼ =

Empty Liposomes.

Figure 5 is a pharmacokinetic comparison of DSPC and HSPC containing liposomal

GI147211 samples performed in Sprague-Dawley rats. See Table 7 for characterization of

5   formulations.

Figure 6 is a pharmacokinetic comparison of free drug and liposomal GI147211

(ALM 993-030) performed in Sprague-Dawley rats. See Table 7 for characterization of

formulation.

Figure 7 is a pharmacokinetic comparison of free drug and liposomal GI147211 (1

10   mg/Kg) given by iv, ip, or subcutaneous routes of administration in mice (n=3, pooled data.

Figure 8 shows the lipid hydrolysis rate for liposomal GI147211 (citric acid) with

and without the addition of ammonium chloride to the final buffer.

Detailed Description of the Invention

Formulations comprising camptothecin encapsulated in a liposome are provided as

15   well as methods of their preparation. The formulations have pharmaceutical uses, including

as anti-tumor or anti-viral agents. In addition, the liposomes have improved

pharmacokinetics, enhanced efficacy as anti-tumor agents, and provide an improved

therapeutic index as compared to the free drug. The formulations include liposomes

comprised of at least one phospholipid and a camptothecin. Additionally, it is also

20   contemplated by this invention to optionally include a sterol, such as cholesterol and/or a

cholesterol analog, in the liposomal formulation. In one embodiment, the formulations

include liposomes comprised of cholesterol, a phosphatidylcholine, an excipient, wherein the

excipient is sulfate or citrate, and a camptothecin. In one embodiment, a portion of the

camptothecin may be precipitated in the aqueous interior of the liposomes by the excipient.

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Further, the formulations described herein are stable upon storage. In one embodiment, the

liposomes are unilamellar vesicles having a size less than 200 nm, most preferably less than

100 nm, wherein the phospholipid is distearoylphosphatidylcholine (DSPC) and includes

cholesterol in a 2:1 molar ratio and the camptothecin is GI147211. In the preferred

5   embodiment, the liposomes are unilamellar vesicles having a size less than 200 nm, most

preferably less than 100 nm, wherein the phospholipid is hydrogenated soy

phosphatidylcholine (HSPC) and includes cholesterol in a 2:1 molar ratio and the

camptothecin is GI147211. In the preferred embodiment, the lipid:camptothecin molar

ratios are 5:1 to 100:1, more preferrably 10:1 to 40:1, and most preferably 15:1 to 25:1.

10   (Lipid includes both phospholipid and cholesterol.) Furthermore, the lipsome have

improved stability relative to free drug and other liposomal formulations of camptothecins.

As used herein, the term "liposome" refers to unilamellar vesicles or multilamellar

vesicles such as are described in U.S. Patent No. 4,753,788, the contents of which are

incorporated herein by reference.

15   "Unilamellar liposomes," also referred to as "single lamellar vesicles," are spherical

vesicles comprised of one lipid bilayer membrane which defines a single closed aqueous

compartment. The bilayer membrane is composed of two layers of lipids; an inner layer and

an outer layer (leaflet). The outer layer of the lipid molecules are oriented with their

hydrophilic head portions toward the external aqueous environment and their hydrophobic

20   tails pointed downward toward the interior of the liposome. The inner layer of the lipid lays

directly beneath the outer layer, the lipids are oriented with their heads facing the aqueous

interior of the liposome and their tails toward the tails of the outer layer of lipid.

"Multilamellar liposomes," also referred to as "multilamellar vesicles" or "multiple

lamellar vesicles," are composed of more than one lipid bilayer membrane, which

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membranes define more than one closed aqueous compartment. The membranes are

concentrically arranged so that the different membranes are separated by aqueous

compartments, much like an onion.

The terms "encapsulation" and "entrapped," as used herein, refer to the incorporation

5   or association of the camptothecin in or with a liposome. The camptothecin may be

associated with the lipid bilayer or present in the aqueous interior of the liposome, or both.

In one embodiment, a portion of the encapsulated camptothecin takes the form of a

precipitated salt in the interior of the liposome. The drug may also self precipitate in the

interior of the liposome.

10   The terms "excipient," "counterion" and "counterion excipient," as used herein, refer

to a substance that can initiate or facilitate drug loading and may also initiate or facilitate

precipitation of the camptothecin in the aqueous interior of the liposome. Examples of

excipients include, but are not limited to, the acid, sodium or ammonium forms of

monovalent anions such as chloride, acetate, lactobionate and formate; divalent anions such

15   as aspartate, succinate and sulfate; and trivalent ions such as citrate and phosphate. Preferred

excipients are citrate and sulfate.

The term "Camptothecin" refers to camptothecin and any and all related analogs or

derivatives thereof which exhibit anti-tumor activity. Camptothecin drugs generally have

the same core ring system. Various modifications or substitutions are found in many

20   camptothecins, preferably such modifications or substitutions are seen in rings A and B. The

camptothecin drugs generally have a similar structure that can exist as lactone and

carboxylate forms as shown below. As used herein, camptothecin refers to both the lactone

and carboxylate forms.
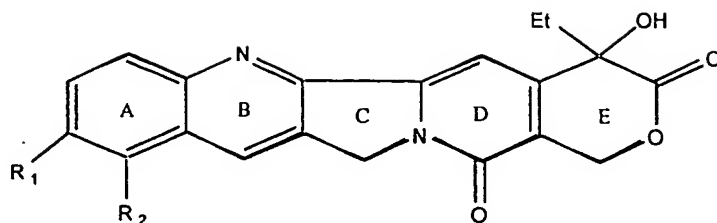
Lactone Form
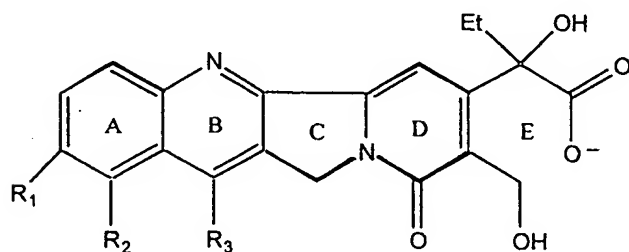
Carboxylate Form

Several examples of camptothecin drugs are provided in **Table 1**.

Other camptothecin drugs have the following structure:
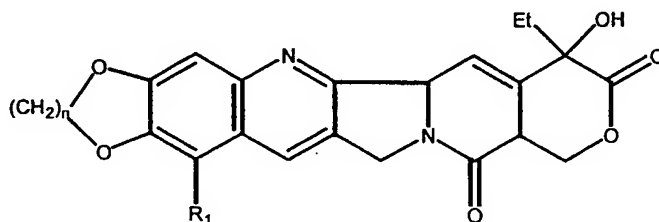
n may be 1-4. Where R_1 is Cl, the drug is 9-chloro-10,11-

methylenedioxycamptothecin; when R_1 is NH_2, the drug is 9-amino-10,11-

methylenedioxycamptothecin; and when R_1 is H, the drug is 10,11-

methylenedioxycamptothecin.

Cmptothecin GI147211C, (7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-

20(S)-camptothecin dihydrochloride) has the following structure

10

• 2HCl
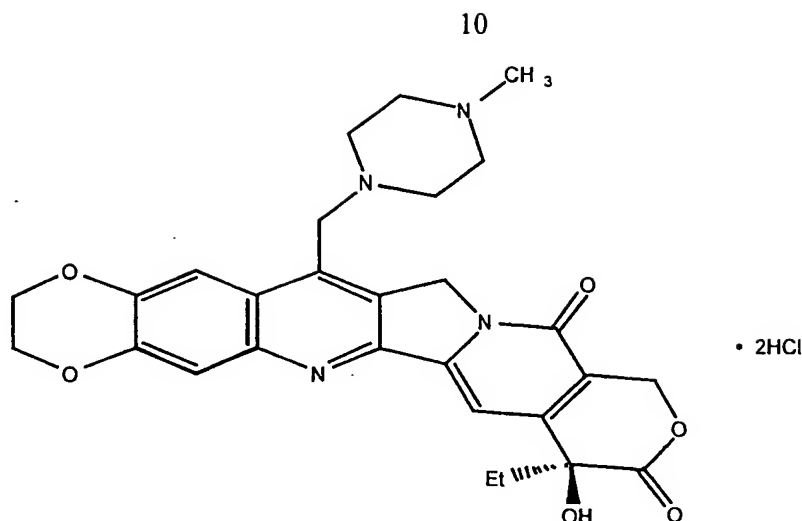
The suffix "C" in GI147211C refers to the dihydrochloride salt, and the suffix "X" in

GI147211X refers to the free base.

The camptothecins may have either "A" and/or "B" ring substitutions. The preferred

5   camptothecins include 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-

camptothecin (GI147211), topotecan, and irinotecan (see **Table 1**), with the most preferred

camptothecin drug being GI147211. Other camptothecins include, but are not limited to, 9-

hydroxycamptothecin, 10-aminocamptothecin, 9-hydroxy-10-dimethylaminomethyl

camptothecin, 20-(RS)-10,11 methylendioxycamptothecin, 9-chloro-10,11-methylenedioxy-

10   (20S)-camptothecin, 7-ethyl-10-hydroxycamptothecin, and 7-ethyl-10-[[[4-(1-piperidino)-1-

piperidino]carbonyl]-oxy]camptothecin.

"Phospholipid" refers to any one phospholipid or combination of phospholipids

capable of forming liposomes. Phosphatidylcholines (PC), including those obtained from

egg, soy beans or other plant sources or those that are partially or wholly synthetic, or of

15   variable lipid chain length and unsaturation are suitable for use in the present invention.

Synthetic, semisynthetic and natural product phosphatidylcholines including, but not

limited to, distearoylphosphatidylcholine (DSPC), hydrogenated soy phosphatidylcholine

(HSPC), soy phosphatidylcholine (soy PC), egg phosphatidylcholine (egg PC),

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hydrogenated egg phosphatidylcholine (HEPC), dipalmitoylphosphatidylcholine (DPPC)

and dimyristoylphosphatidylcholine (DMPC) are suitable phosphatidylcholines for use in

this invention. All of these phospholipids are commercially available. Preferred PCs are

HSPC and DSPC; the most preferred is HSPC.

5   Further, phosphatidylglycerols (PG) and phosphatic acid (PA) are also suitable

phospholipids for use in the present invention and include, but are not limited to,

dimyristoylphosphatidylglycerol (DMPG), dilaurylphosphatidylglycerol (DLPG),

dipalmitoylphosphatidylglycerol (DPPG), distearoylphosphatidylglycerol (DSPG)

dimyristoylphosphatidic acid (DMPA), distearoylphosphatidic acid (DSPA),

10   dilaurylphosphatidic acid (DLPA), and dipalmitoylphosphatidic acid (DPPA).

Distearoylphosphatidylglycerol (DSPG) is the preferred negatively charged lipid when

used in formulations. Other suitable phospholipids include phosphatidylethanolamines,

phosphatidylinositols, and phosphatidic acids containing lauric, myristic, stearoyl, and

palmitic acid chains. Further, incorporation of polyethylene glycol (PEG) containing

15   phospholipids is also contemplated by the present invention.

The term "parenteral" as used herein refers to intravenous (IV), intramuscular (IM),

subcutaneous (SubQ) or intraperitoneal (IP) administration.

The term "improved therapeutic index" refers to a higher therapeutic index relative

to the free drug. The therapeutic index is expressed as a ratio of the lethal dose for 50% of

20   the animals relative to the effective dose.

It is contemplated by this invention to optionally include cholesterol in the

liposomal formulation. Cholesterol is known to improve liposome stability and prevent

loss of phospholipid to lipoproteins *in vivo*.

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Any lipid:camptothecin ratio that is efficacious is contemplated by this invention.

Preferred lipid:camptothecin molar ratios are 5:1 to 100:1, more preferably 10:1 to 40:1.

The most preferred lipid:camptothecin molar ratios are 15:1 to 25:1. Preferred liposomal

formulations include phospholipid:cholesterol molar ratios over the range of 1.5:0.5 to 2:1.5.

5   Most preferred liposomal formulation is 2:1 PC:chol with or without 1 to 4 mole percent PG.

The most preferred liposomal size is less than 100 nm. The preferred loading effciency of

drug is a percent encapsulated camptothecin of about 70% or greater. Encapsulation

includes molecules present in the interior aqueous space of the liposome, molecules in the

inner or outer leaflet of the membrane bilayer, molecules partially buried in the outer leaflet

10   of the bilayer and partially external to the liposome, and molecules associated with the

surface of the liposome, e.g., by electrostatic interactions.

Generally, the process of preparing the formulation embodied in the present

invention is initiated with the preparation of a solution from which the liposomes are

formed. This is done, for example, by weighing out a quantity of a phosphatidylcholine,

15   optionally cholesterol and optionally a phosphatidylglycerol and dissolving them in an

organic solvent, preferably chloroform and methanol in a 1:1 mixture (v/v) or alternatively

neat chloroform. The solution is evaporated to form a solid lipid phase such as a film or a

powder, for example, with a rotary evaporator, spray dryer or other means. The film or

powder is then hydrated with an aqueous solution containing an excipient having a pH range

20   from 2.0 to 7.4 to form a liposome dispersion. The preferred aqueous solution for purposes

of hydration is a buffered solution of the acid, sodium or ammonium forms of citrate or

sulfate. The preferred buffers are > 5mM, more preferably 50 mM, citric acid (pH 2.0 - 5.0),

ammonium citrate (pH 2.0 - 5.5), or ammonium sulfate (pH 2.0 to 5.5). It would be known

by one of skill in the art that other anionic acid buffers could be used, such as phosphoric

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acid. The lipid film or powder dispersed in buffer is heated to a temperature from about

25°C to about 70°C depending on the phospholipids used.

Multilamellar liposomes are formed by agitation of the dispersion, preferably through

the use of a thin-film evaporator apparatus such as is described in U.S. Patent No. 4,935,171

5   or through shaking or vortex mixing. Unilamellar vesicles are formed by the application of a

shearing force to an aqueous dispersion of the lipid solid phase, e.g., by sonication or the use

of a microfluidizing apparatus such as a homogenizer or a French press. Shearing force can

also be applied using either injection, freezing and thawing, dialyzing away a detergent

solution from lipids, or other known methods used to prepare liposomes. The size of the

10   liposomes can be controlled using a variety of known techniques including the duration of

shearing force. Preferably, a homogenizing apparatus is employed to form unilamellar

vesicles having diameters of less than 200 nanometers at a pressure of 3,000 to 14,000 psi,

preferably 10,000 to 14,000 psi and a temperature of about the aggregate transition

temperature of the lipids.

15   Unentrapped excipient is removed from the liposome dispersion by buffer

exchange to 9% sucrose using either dialysis, size exclusion column chromatography

(Sephadex G-50 resin) or ultrafiltration (100,000 - 300,000 molecular weight cut off).

Each preparation of small unilamellar liposomes is then actively loaded with GI147211 or

other camptothecin, for approximately 10 - 30 minutes against a gradient, such as a

20   membrane potential, generated as the external pH is titrated to the range of 5.0 to 6.5 with

sodium hydroxide. The temperature ranges during the drug loading step is generally

between 50 - 70°C with lipid:drug ratios between 5:1 to 100:1. Unentrapped camptothecin

is removed from the liposome dispersion by buffer exchange to 9% sucrose using either

dialysis, size exclusion column chromatography (Sephadex G-50 resin) or ultrafiltration

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(100,000 - 300,000 molecular weight cut off). Samples are generally filtered at 55-65 °C

through a 0.22 micron filter composed of either cellulose acetate or polyether sulfone.

As described above, the camptothecin is generally loaded into pre-formed

liposomes using known loading procedures (see for example Deamer *et al*. BBA 274:323-

5   335 (1972); Forssen U.S. Patent No. 4,946,683; Cramer *et al*. BBRC 75:295-301 (1977);

Bally U.S. Patent No. 5,077,056). The loading can be by gradient or concentration

loading, such as pH gradients or ammonium gradients. If a pH gradient is used, it is

preferable to begin with an internal pH of approximately pH 2-3. The excipient is the

counterion in the loading process and when it comes in contact with the camptothecin in

10   the interior of the liposome, the excipient may cause a substantial portion of the

camptothecin to precipitate. The drug may also self precipitate in the interior of the

liposome. This precipitation may protect the lactone ring of the camptothecin from

hydrolysis. An excipient, such as citrate or sulfate, may precipitate the camptothecin and

can be utilized in the interior of the liposomes together with a gradient (pH or ammonia) to

15   promote camptothecin loading.

Drug loading by pH gradient usually includes a low pH in the internal aqueous space

of the liposomes, and this internal acidity is incompletely neutralized during the drug loading

process. This residual internal acidity can cause chemical instability in the liposomal

preparation (e.g., lipid hydrolysis), leading to limitations in shelf life. To quench this

20   residual internal acidity, membrane permeable amines, such as ammonium salts or alkyl-

amines can be added following the loading of the camptothecin in an amount sufficient to

reduce the residual internal acidity to a minimum value. Ammonium salts that can be used

include ones having mono-or multi-valent counterions, such as, but not limited to,

ammonium sulfate, ammonium hydroxide, ammonium acetate, ammonium chloride,

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ammonium phosphate, ammonium citrate, ammonium succinate, ammonium lactobionate,

ammonium carbonate, ammonium tartrate, and ammonium oxalate. The analogous salt of

any alkyl-amine compound which is membrane permeable can also be used, including, but

not limited to, methylamine, ethylamine, diethylamine, ethylenediamine, and propylamine.

5   The therapeutic use of liposomes can include the delivery of drugs which are

normally toxic in the free form. In the liposomal form, the toxic drug may be directed away

from the sensitive tissue where toxicity can result and targeted to selected areas where they

can exert their therapeutic effects. Liposomes can also be used therapeutically to release

drugs slowly, over a prolonged period of time, thereby reducing the frequency of drug

10   administration through an enhanced pharmacokinetic profile. In addition, liposomes can

provide a method for forming an aqueous dispersion of hydrophobic drugs for intravenous

delivery.

The route of delivery of liposomes can also affect their distribution in the body.

Passive delivery of liposomes involves the use of various routes of administration e.g.,

15   parenterally, although other effective administration forms, such as intraarticular injection,

inhalant mists, orally active formulations, transdermal iotophoresis or suppositories are also

envisioned. Each route produces differences in localization of the liposomes.

The invention also provides a method of inhibiting the growth of tumors, both drug

resistant and drug sensitive, by delivering a therapeutic or effective amount of liposomal

20   camptothecin to a tumor, preferably in a mammal. Because dosage regimens for

camptothecin are well known to medical practitioners, the amount of the liposomal

camptothecin formulations which is effective or therapeutic for the treatment of the above

mentioned diseases or conditions in mammals and particularly in humans will be apparent

to those skilled in the art. The optimal quantity and spacing of individual dosages of the

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formulations herein will be determined by the nature and extent of the condition being

treated, the form, route and site of administration, and the particular patient being treated,

and such optimums can be determined by conventional techniques. It will also be

appreciated by one of skill in the art that the optimal course of treatment, i.e., the number of

5   doses given per day for a defined number of days, can be ascertained by those skilled in the

art using conventional course of treatment determination tests

          Inhibition of the growth of tumors associated with all cancers is contemplated by

this invention, including multiple drug resistant cancer. Cancers for which the described

liposomal formulations may be particularly useful in inhibiting are ovarian cancer, small

10   cell lung cancer (SCLC), non small cell lung cancer (NSCLC), colorectal cancer, breast

cancer, and head and neck cancer. In addition, it is contemplated that the formulations

described and claimed herein can be used in combination with existing anticancer

treatments. For example, the formulations described herein can be used in combination

with taxanes such as 1) Taxol (paclitaxel) and platinum complexes for treating ovarian

15   cancer; 2) 5FU and leucovorin or levamisole for treating colorectal cancer; and 3) cisplatin

and etoposide for treating SCLC.

          This invention will be more fully understood by reference to the following examples,

which are intended to be illustrative of the invention, and not limiting thereof. Example 1

describes the pharmacokinetics of liposomal formulations of GI147211 prepared by three

20   different liposome loading techniques. Example 2 describes liposomal formulations of

GI147211 prepared by gradient loading and the use of ammonia to quench the liposome

internal acidity. Example 3 describes precipitation of GI147211 salts. Example 4 describes

the concentration dependence of precipitation of GI147211 salts using selected excipients.

Example 5 describes the *in vitro* efficacy screening and *in vivo* pharmacokinetics of

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liposomal formulations of GI147211. Example 6 describes the *in vivo* antitumor efficacy of

liposome encapsulated GI147211 in comparison to free drug. Example 7 describes the

therapeutic index determination of GI147211, topotecan and liposomal formulations of

GI147211 in two separate xenograft models. Example 8 describes repeat dose efficacy

5   studies of liposomal formulations of GI147211 compared to free GI147211 at equally toxic

doses. Example 9 compares two different liposomal preparations of GI147211 and free

GI147211 in a repeat dose study in the KB tumor xenograft model.

Example 1

Pharmacokinetics of membrane, passively and actively loaded liposomal

10   **formulations of GI147211**

Liposomal GI147211 samples were prepared by three different loading techniques:

entrapment of the drug in the liposome bilayer, passive entrapment and by active loading

against a membrane potential generated by a pH gradient. The pharmacokinetics were

then compared for free drug and membrane entrapped GI147211 liposomes dosed at 5

15   mg/kg in Sprague Dawley rats. Rats were dosed at 1 mg/kg to compare free GI147211 to

passive and actively loaded liposomes.

Membrane loaded GI147211 liposomes were prepared by first cosolubilizing

phospholipid (DSPC), cholesterol and GI147211 at a lipid:drug ratio of about 10:1 (w/w)

in organic solvent. The solution was dried down to a thin film using nitrogen gas and

20   elevated temperature then stored in a vacuum desiccator under reduced pressure until use.

Lipid films were rehydrated with an aqueous solution, typically 9% sucrose and 10mM

sodium succinate, pH 5.4 in sufficient volume that the lipid concentration was about 50

mg/ml and the GI147211 concentration was about 5 mg/ml. Samples were then sonicated

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for 10-15 minutes above the aggregate lipid phase transition temperature until solutions

were translucent in appearance, then filtered through a 0.22 micron filter.

Passively loaded GI147211 liposomes comprised of negative (DSPG) and/or

neutrally charged lipids (DSPC) and cholesterol were prepared as follows. Aqueous

5   solutions of GI147211C were prepared at drug concentrations of about 30 mg/ml drug by

dissolving the drug in a 9% sucrose and 50mM citric acid solution, pH 2.2 at 65°C. Lipid

films or spray dried powders were prepared by cosolubilizing the lipid components in an

organic solvent system, then drying the solution down to a film or powder using nitrogen

gas and elevated temperature. Lipid films or powders were then hydrated at 150 mg/mL

10   lipid to a lipid to drug ratio of 5:1 by adding the drug solutions described above, mixing

and heating at about 65°C. Samples were then sonicated for about 10-15 minutes above

the aggregate lipid phase transition temperature until solutions were translucent in

appearance. Unentrapped GI147211 was separated from the liposome encapsulated drug

using a Sephadex G-50 column eluted with 9% sucrose. The resultant liposomal

15   GI147211 formulations were concentrated to the desired final drug concentration and the

pH was adjusted to about 5.6-5.7.

Actively loaded and actively loaded/ammonia quenched samples were prepared as

described in **Example 2**.

Particle size diameters were measured to be less than 100 nm using the MicroTrac

20   Ultrafine Particle Analyzer for all small unilamellar vesicles described above with the

exception of the membrane loaded sample which had a bimodal size distribution with

7.7% of the distribution in the main peak with a median diameter less than 100 nm..

As shown in **Table 2A**, when the pharmacokinetics of free drug are compared to

membrane entrapped drug dosed at 5 mg/kg in rats, there is a minimal (3-fold)

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improvement observed in the area under the curve (AUC), maximum concentration

measured at 15 minutes(C_{max}) (2.5 fold), a two-fold decrease in the clearance rate (CL) and

a one hour extension in the half life ($t_{1/2}$) for the liposomal samples. **Table 2B** shows the

results of passively loaded liposomal GI147211 sample, prepared with and without PG,

5   compared to free drug dosed at 1 mg/kg in rats. There is an 110-140 fold increase in AUC

and C_{max}, 120-140 fold decrease in clearance rate, and identical half life for liposomal drug

compared to free drug. **Table 2B** also illustrates that there is an additional order of

magnitude improvement in the AUC and reduction in clearance rate in the liposomal

GI147211 samples that were actively loaded against citric acid or ammonium sulfate and

10   in one case quenched with ammonium chloride after loading compared to passive loading.

800-1200 fold increases in AUC, 200 fold increases in C_{max}, 600-1200 fold decreases in

clearance rates, and half life extensions ranging from an additional 2 to 7 hours were

observed for actively loaded GI147211 liposomes compared to free drug. This study

demonstrates that the preferred loading method to achieve the best retention of drug with

15   liposomes *in vivo* is active loading in the presence of a counterion which may precipitate

some drug in the internal aqueous core of the liposomes.

Example 2

Liposomal Formulations of GI147211

Phospholipids and cholesterol used herein were obtained as dry powders from

20   Avanti Polar Lipids, Nippon, Lipoid or Sygena and were used without further purification.

All other chemicals were reagent grade and were used without further purification.

For each liposome preparation described below, solutions of different counterion

excipients were prepared and entrapped in the internal aqueous core of liposomes, then a

water-soluble camptothecin derivative was added to the outside of the liposomes and

loaded against a membrane potential generated by a pH or ion concentration difference

between the inside and outside of the liposomes to enhance drug loading.

First, lipid films or spray dried powders containing various phospholipids

including phosphatidylcholine, cholesterol and phosphatidylglycerol were prepared by

5   cosolubilizing the lipid components in an organic solvent system, then drying the solution

down to a film or powder using nitrogen gas and elevated temperature. Different

phospholipid sources (synthetic, semi-synthetic, egg, soy), chain length (14 - 18 carbons)

and degree of unsaturation (1 to 4 double bonds) were explored in the range of molar ratios

as shown in **Table 3**. Each lipid powder or film was hydrated at lipid concentrations of

10   100 - 150 mg/ml with an aqueous solution containing a counterion solution for

precipitation (acid, sodium or ammonium forms of citrate, sulfate, succinate, phosphate,

formate, aspartate or lactobionate), in the pH range of 2.0 to 7.4. Small unilamellar

liposomes (<100 nm, median diameter using the MicroTrac Ultrafine Particle Analyzer)

were then formed from these mixtures at temperatures above the respective lipid phase

15   transitions (~40-70°C) using shear force applied with either sonication or alternatively with

a homogenizer at 10,000 - 14,000 psi. Excipient that was not entrapped in the aqueous

core of the liposomes was removed from the liposome dispersion generally by buffer

exchange to 9% sucrose using dialysis, size exclusion column chromatography (Sephadex

G-50 resin) or ultrafiltration (100 kD - 300 kD molecular weight cut off). Each

20   preparation of small unilamellar liposomes was then actively loaded with GI147211 for

approximately 10 - 30 minutes against a membrane potential generated as the external pH

was titrated to the range of 5.0 to 6.5 with sodium hydroxide or other suitable base. The

temperature ranges during the drug loading step were generally between 50 - 70°C at

lipid:drug ratios between 5:1 to 100:1. Unentrapped drug was removed from the liposome

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dispersion by buffer exchange to 9% sucrose using dialysis, size exclusion column

chromatography (Sephadex G-50 resin) or ultrafiltration using a 100 kD - 300 kD

molecular weight cut off membrane. Samples were filtered at 55-65 °C through a 0.22

micron filter composed of either cellulose acetate or polyether sulfone. Results of

5   characterization are shown below in **Tables 3, 4, 5 and 7**.

Although this example describes liposomal preparations of GI147211, it would be

known by one of skill in the art that liposomal preparations of other camptothecins can

also be prepared as described.

Ammonia quench

10   In the formulations described above, gradient methods were used to load the

camptothecin derivative GI147211C into liposomes. The setup of the gradients includes a

low pH in the internal aqueous space of the liposomes, and this internal acidity may be

incompletely neutralized during drug loading. Any residual internal acidity can cause

chemical instability in the liposomal preparation (e.g., lipid hydrolysis), leading to

15   limitations in shelf life. Residual internal acidity was quenched in certain liposomal

formulations as follows.

410mL of a solution containing liposomes composed of 2:1 HSPC:Cholesterol and

encapsulating a 50 mM citric acid buffer at a total lipid concentration of 58.6 mg/mL, was

heated to 55°C. To the liposome solution was added 60 mL of GI147211C stock solution

20   at concentration of 20 mg/ml at a temperature of 55°C. The pH of the solution was titrated

from 2.5 to about 4.4 by adding approximately 3 mL of 2.5M NaOH. The pH was then

carefully titrated to 5.8 with 1 M NaOH. Following heating for approximately 20 minutes

from the first base addition, the solution was then cooled to below 35 °C and ultrafiltered

against 5 L of pH 6.5 dialysis solution containing 10mM NH_4Cl, 9% w/w sucrose. At the

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end of ultrafiltration, the solution pH was adjusted to 6.5, diluted to target concentrations

and filtered through a 0.2μm pore size filter.

Liposomal GI147211 samples so treated exhibited markedly reduced rates of lipid

hydrolysis, and thus are rendered more chemically stable relative to untreated samples.

5   Generation of suitably stable liposomal samples with consistent control over lipid

hydrolysis rates is required to enhance sample shelf life as a liquid. This is generally a

concern for any liposome which is loaded at pH values that differ significantly from

neutral, and in this case was investigated specifically in reference to liposomal GI147211

formulations.

10   **Figure 8** shows accelerated condition (25°C) lipid hydrolysis results for a

liposomal GI147211 sample prepared with 100 mM citric acid at hydration, followed by

loading at a target 20:1 lipid to drug ratio. Following drug loading, the solution was split

in half with a control solution and a second sample where ammonium chloride was added.

The lipid hydrolysis rate for the sample with ammonium chloride added is dramatically

15   reduced by about 167-fold in comparison to the control system.

Pharmacokinetics and efficacy studies comparing ammonium quenched versus

non-quenched liposomal GI147211 samples revealed identical PK and efficacy profiles

(see **Table 2B and Table 15**). Thus, ammonium chloride has no deleterious effect on the

liposomal formulation while providing improved chemical stability for phospholipids.

Example 3

Precipitation of GI147211 Salts

Several excipients have been identified which may induce precipitation of

GI147211 from aqueous media. The excipients include, but are not limited to, the acid,

5   sodium or ammonium forms of monovalent anions such as chloride, acetate, lactobionate

and formate; divalent anions such as succinate, aspartate and sulfate; and trivalent ions

such as citrate and phosphate. To assess the relative degree of precipitation brought about

with each excipient, 5 mg/ml solutions of GI147211 were prepared at low pH (2 ± 0.5) and

different counterions were added. The solutions of GI147211 and counterion were

10   allowed to stand for 15 to 20 minutes, then centrifuged at 3600 rpm for 10 minutes to

isolate the precipitate. The quantity of GI147211 remaining in solution for each sample

was then assessed by assaying the supernatants using HPLC with the following conditions:

C18 ODS Hypersil column from Keystone, mobile phase of 30/70 (v/v) acetonitrile/0.1%

acetic acid + 0.1% aqueous triethylamine buffer, pH 3.30 with UV detection at 270 nm.

15   The amount of GI147211 precipitated was also tabulated by the taking the difference of

the concentration of the remaining soluble fraction from 5mg/ml, the original GI147211

concentration in solution. Each number is expressed in **Table 6** as percentage of the

original solution concentration of 5 mg/ml. In some instances, higher counterion

concentration might induce more precipitation than noted in **Table 6**. Sulfate, citrate, and

20   succinate cause greater than 97% of the drug to precipitate; chloride and phosphate induce

precipitation of greater than 82% of the drug; formate and aspartate result in greater than

55% of the drug precipitated; and, sucrose and lactobionate do not cause appreciable

precipitation, less than 10%, of GI147211 at the counterion concentrations tested. The

precipitate will be composed of the closed lactone ring form of the camptothecin

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derivative as the solution pH of these experiments was approximately 2. "A" ring

substituted water soluble camptothecin derivatives such as topotecan, irinotecan (CPT-11)

and 9-aminocamptothecin may also display the same degree of precipitation in the

presence of the counterions listed above.

5   **Example 4**

Concentration Dependence of Precipitation of GI147211 Salts Using Selected

Excipients

       To more fully explore the concentration dependence of GI147211 precipitation, 5

mg/ml solutions of the drug were prepared as described in **Example 3** and titrated with

10   increasing concentrations of the counterion excipients sulfate, citrate, phosphate and

chloride. The solutions of GI147211 and counterion were allowed to stand for 15 to 20

minutes, then centrifuged at 3600 rpm for 10 minutes to isolate the precipitate and the

supernatants were assayed as described in **Example 3**. **Figure 1** details the concentration

dependence of the precipitation that was observed. Sulfate and citrate are the most

15   efficient and preferred excipients for drug precipitation, reducing the fraction of drug

remaining in solution at lower concentrations than chloride or phosphate with no time

dependence. Liposomal samples have been prepared by active loading utilizing the

counterions listed above and shown in **Table 7**. Percent loading of drug varied with

counterion from 13% (NaCl), 61% (phosphate) to 75% and 89%, respectively for

20   ammonium sulfate and citric acid.

       In a separate study, precipitation of the camptothecin GI147211 was examined at

the predicted concentration in the interior of the liposome (approx. 40 mg/ml camptothecin

GI147211) after loading in the presence of 50 mM ammonium sulfate. The results show

that only 0.5% of the camptothecin GI147211 remained in solution, essentially 99% of the

25

drug had precipitated. A similar precipitation of drug is anticipated in the interior of the

liposomes. Self precipitation of the drug may also be anticipated in the interior of the

liposomes.

Also, shown in **Figure 2** are stability points for liposomal camptothecin GI147211

5   samples containing either citrate or sulfate counterions. The GI147211 losses were

observed to be 4% for the sulfate or citrate sample after storage at 2-8°C for 80-90 days,

and typical precision for this type of HPLC assay is 3%.

Example 5

10   *In Vitro* **Efficacy Screening and** *in Vivo* **Pharmacokinetics of Liposomal GI147211**

Formulations

Liposomal formulations were prepared as described in **Example 2** and samples were

characterized as shown in **Table 7** below and tested for *in vitro* efficacy screening and *in*

vivo performance of liposomal GI147211.

15

In Vitro Efficacy Screening

In vitro efficacy screening has been performed using GI147211 and liposomal

formulations of GI147211. The purpose of this *in vitro* experimentation was to ensure that

liposomal encapsulation of GI147211 did not result in decreased activity relative to non-

20   liposomal compound. Standard tumor cell-based cytototoxicity assays assessing ^{3}H-

thymidine incorporation were performed.

Tumor cells were seeded into 96-well tissue culture plates (1×10^4 cells/well) $\cong$4h

prior to experimentation. Serial dilutions (1:10 dilutions, 10^5 to 10^{-3} ng/ml, n=8

wells/dilution) of GI147211, liposomal GI147211 and TP treatments were prepared

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(immediately before use) and added to cell containing plates. Positive and negative (Tris treated) control groups were included on each assay plate. Tumor cells were then labeled with 0.25μCi of [methyl-^{3}H] thymidine and incubated for 42h under tissue culture conditions (37°C, 5% CO_2, 100% relative humidity), except for C6, U251, A673 and B16-

5   F1 cells which were incubated for 24h. Cells were then lysed, harvested onto glass-fiber filters and unincorporated [methyl-^{3}H] thymidine removed by filter washing. Filters were processed for scintillation counting and cpm-^{3}H/well determined. Data (on a cell-type by cell-type basis) were normalized to control thymidine incorporation, fitted to a standard 4-parameter non-linear regression equation and IC_{50} values (50% Inhibitory Concentration)

10   determined for each treatment. Statistical differences were determined by Rank Analysis of Variance with multiple comparisons (no adjustment for multiplicity of testing) to test each cell line for differences in the IC_{50} estimates between cells treated with liposomal GI147211, GI147211, and topotecan. IC_{50} data are presented for each treatment group (median, minimum, and maximum) with significance indicated ($p < 0.05$). Data are

15   presented from multiple sets of experimentation, involving multiple liposomal formulations of GI147211.

In addition, GI147211 and liposomal GI147211 activities were also compared to that of the commercially available topoisomerase I inhibitor topotecan. The data (**Table 8** and **Figures 3A-3D**) produced by this experimentation suggests that liposomal

20   formulations of GI147211 and free GI147211 are significantly more potent than topotecan, for the majority of tumor cell lines investigated ($p < 0.05$, all cases). In addition, data for several tumor cell lines support the possibility that liposomal formulations of GI147211 may be more potent than GI147211 free-drug in some cases ($p < 0.05$ as indicated in **Table 8**). Data presented in tabular form represent results from a large number (n>100) of

27

cytotoxicity experiments, involving multiple lots of liposomal GI147211 produced during

formulation development. Consequently, IC_{50} values are presented as median values

(ng/ml) with indicated maxima and minima. In addition, representative data are provided

(**Figures 4A-4D**) demonstrating the consistency of tabular data with results generated

5   using a preferred formulation. Further, data provided (**Figure 4A-4D**) indicate that drug-

free empty liposome vesicles do not induce appreciable cytotoxicity, supporting the

contention that observed activity of liposomal GI147211 is due to drug and not liposomal

components. These data support liposomal GI147211 as an active and effective

chemotherapeutic agent with the potential for increased activity relative to free GI147211.

10

In Vivo Pharmacokinetic Studies

        Pharmacokinetic studies were carried out on various liposomal formulations of

GI147211 in rats and mice by IV bolus administration. Plasma samples were precipitated

with acetonitrile and the supernatant for each sample was analyzed by reverse phase HPLC

15   using isocratic elution and monitoring fluorescence. Standards and quality control

samples were analyzed in a similar manner. Concentration values were determined for

unknown samples and quality control samples based on interpolation using a linear fit of

the standard samples. In each study the average plasma concentration at each time point

was calculated and utilized in the pharmacokinetic analysis. Noncompartmental analysis

20   were carried out using WinNonlin™ (Standard Edition, version 1.5 ©, Scientific

Consulting, Inc.) and the following parameters were calculated: the maximum

concentration extrapolated at zero time (Cmax), the area under the curve from zero to

infinite time (AUC total), the terminal phase half life (t1/2), the clearance rate (Cl), and the

volume of distribution at steady state (Vss).

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Table 9 summarizes the plasma pharmacokinetic parameters determined from

several of these studies. In all cases the liposomal formulations show a significant

increase in total exposure relative to free drug administered at a similar dose as determined

by AUC total. This result was consistent in studies conducted in both rats and mice. The

5   increase in AUC total for actively loaded liposomal formulations compared with free drug

ranged from 473 to 1173-fold. The majority of the increase in exposure comes from a

decrease in the volume of distribution at steady state for the liposomal formulations that

approaches the plasma volume. These data support a model where the liposomal

formulations reside primarily within the plasma volume and are cleared with a terminal

10   half life of 3 to 8 hours depending on the formulation.

Dose dependence on plasma pharmacokinetics was investigated for two liposomal

formulations NA-908-73 (sulfate counterion) and NA943-072B (citrate counterion). In

both studies, dose dependence appeared to be linear as a function of dose as shown by

similar clearance rates across the dose ranges tested for a given formulation.

15   The citrate and sulfate formulations with either 2:1 molar ratio of HSPC or

DSPC:cholesterol are preferred formulations based on increased efficacy data and

pharmacokinetic properties. **Figure 5** shows the plasma concentrations as a function of

time following administration in Sprague-Dawley rats, dose 1 mg/kg. Studies on one

liposomal GI147211 formulation (NA943-072B), performed approximately 3 months

20   apart, show that the pharmacokinetics does not change after liposomal drug has been

stored for greater than 3 months at 2-8°C. Furthermore, citrate formulations prepared with

either HSPC or DSPC show highly similar plasma pharmacokinetics. **Figure 6** shows the

plasma pharmacokinetics of a formulation (2:1 HSPC:cholesterol, loaded with 50 mM

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citric acid and quenched with NH₄Cl) in comparison with free drug in Sprague-Dawley

rats, dose 1 mg/kg. The AUC relative to free drug is approximately 978.

Routes of administration and corresponding plasma pharmacokinetics were

evaluated with GI147211 free drug and liposomal formulation NA-908-73 (see **Table 7**

5   for formulation characteristics) in mice. Animals received a 1 mg/kg dose by intravenous

(I.V.) intraperatoneal (I.P.) or subcutaneous administration and plasma samples were

collected over 24 hours. **Figure 7** shows the plasma concentrations for free drug and

liposomal GI147211 as a function of time and the plasma pharmacokinetics are

summarized in **Table 16**. Comparison of exposure determined by AUC between free drug

10   and liposomal showed increases of 190 to 500 fold for the three routes of delivery. This

study further supports the advantage of liposomal GI147211 formulations to increase

plasma circulation time and that additional routes of delivery may be utilized to maintain

high concentrations in the plasma.

15   **Example 6**

In Vivo **Antitumor Efficacy of Liposome Encapsulated GI147211 vs Free GI147211**

The antitumor efficacy of liposomal formulations of GI147211 was demonstrated

in several human tumor xenograft models. In all the studies performed, subcutaneous

tumors implanted on the anterior flank of nude mice were allowed to grow to an

20   established size of 200 +/- 50 mm³ prior to dosing drugs.

In vivo efficacy studies have been performed to compare the activity of the free

GI147211 to the liposome formulated drug. When dosed identically, the liposomal

formulation of GI147211 was shown to be more potent and efficacious in reducing tumor

growth (**Table 10**). The xenograft models included the HT29 and SW48 human colon

30

tumor lines, and the KB and KBV head-neck tumors. Drugs were dosed at either 6 or

9mg/kg intravenously, on a weekly schedule for 3 consecutive weeks. In the SW48

xenograft study, the animals received a total of two cycles of therapy (6 injections), with a

10 day recovery period separating the two therapeutic courses. In the HT29 xenograft

5   study a 12 and 14mg/kg dose group was added. In this experiment, the liposomal

GI147211 dosed animals in the high dose groups received only 2 consecutive doses of

drug, while the free GI147211 groups were dosed for all three weeks. In both the KB and

KBV studies, the liposomal GI147211 appeared to be more efficacious than free drug

alone. This is illustrated by both the magnitude of response as well as the duration. This

10   was particularly significant in the multiple drug resistant (MDR+) tumor line KBV, where

the free drug had little effect on tumor growth, but the liposomal GI147211 demonstrated a

dose dependent inhibition of tumor growth. In the two colon tumor xenograft studies, the

differences were less pronounced initially, and only apparent in the SW48 tumor study

after the second round of treatment. In the HT29 study the difference in tumor response

15   was more dramatic in that the lower 6mg/kg doses of liposomal GI147211 were as

efficacious as the high dose, 14mg/kg, free GI147211. When compared at identical dose

and schedule in the SW48 and HT29 colon tumor models, liposomal GI147211 produced

95% tumor growth inhibition in both models compared to 86% and 54% produced by free

drug. A more striking difference was seen in the KB tumor model where liposomal

20   GI147211 demonstrated a Log 10 Cell Kill index of 7.13, compared to 1.64 for free drug.

In addition, liposomal GI147211 produced 65% tumor growth inhibition in the MDR+

tumor model KBV, whereas the free drug was essentially inactive. When dosed at equally

toxic levels, liposomal GI147211 was still more efficacious than free drug alone.

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Example 7.

Therapeutic Index Determination of GI147211, Topotecan and a Liposomal

Formulation of GI147211 in Two Separate Xenograft Models.

Single dose toxicity and efficacy experiments of a liposomal formulation of

5   GI147211, GI147211 and topotecan were performed for purposes of determining

differences in therapeutic index. The established tumor xenograft models used in these

studies included the KB head and neck tumor and the ES2 ovarian tumor. All test groups

consisted of 10 nude mice, and the drugs were delivered as a single intravenous dose bolus

injection via the tail vein. Topotecan was dosed from 6 to 40mg/kg, GI147211 was dosed

10   from 6-30mg/kg, and liposomal GI147211 was dosed from 3 to 40 mg/kg. The therapeutic

index was determined on day 27 post dose, by dividing the LD50 by either the ED60 or

ED80. Results from both studies demonstrated that liposomal GI147211 has a consistent

increase in the therapeutic index ranging from 3 to 14 fold over that of free GI147211,

Table 11.

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Example 8.

Repeat Dose Efficacy Studies of Liposomal GI147211 Compared to Free GI147211 at Equal Toxic Doses

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Several repeat dose efficacy studies comparing liposomal GI147211 to free

GI147211 at equal toxic doses were performed with the KB, KBV and ES2 tumor

xenograft models. Drugs were dosed on days 1,8 and 15. In all three model systems

liposomal GI147211 demonstrated enhanced tumor growth delay compared to free

25   GI147211 when dosed at equal toxic levels, **Tables 12-14.**

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Example 9.

Comparison of HSPC vs. DSPC Based Liposomal GI147211 Preparations and Free

GI147211 in a Repeat-Dose Study in the KB Tumor Xenograft Model

      A dose response study was performed in the KB tumor xenograft model where two

5   different preparations of liposomal GI147211 were compared to free GI147211 following

intravenous administration. On a day 1 and day 8 dose schedule and after two weeks rest

the same cycle of therapy was repeated with doses delivered on day 23 and day 30. All

animals in the liposomal GI147211 groups received a total of 4 drug doses, whereas the

control group and the GI147211 group only received two drug doses due to excessive

10   tumor growth. The results are shown in **Table 15,** and clearly demonstrate that at equal

toxic doses there were no apparent differences between the two liposomal GI147211

formulations, but the GI147211 group was significantly less efficacious as an antitumor

agent. The difference between the two liposomal GI147211 preparations tested was that

HSPC was used in place of DSPC in batch ALM993-028 (see **Table 7** for details

15   regarding the formulations).

      The invention claimed herein has been described with respect to particular

exemplified embodiments. However, the foregoing description is not intended to limit the

invention to the exemplified embodiments, and the skilled artisan should recognize that

20   variations can be made within the scope and spirit of the invention as described in the

foregoing specification. The invention includes all alternatives, modifications, and

equivalents that may be included within the true spirit and scope of the invention as defined

by the appended claims.

Table 1.
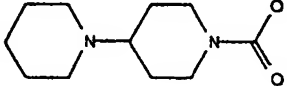
Camptothecin drugs	R_1	R_2	R_3
camptothecin	H	H	H
9-aminocamptothecin	H	NH_2	H
9-nitrocamptothecin	H	NO_2	H
10-hydroxycamptothecin	OH	H	H
Topotecan (Hycamtin™)	OH	$CH_2NH(CH_3)_2$	H
Irinotecan		H	CH_2CH_3

Table 2A. Comparative Rat Pharmacokinetics Following 5mg/kg IV dose of Free or Liposomal GI147211C

Lot #	Lipid	Counterion	Loading	AUC (µg*hr/ml)	C_{max} (0.25 hr) (µg/ml)	$t_{1/2}$ (hr)	CL (L/kg/hr)
SMC 880-09C	-	-	free drug	0.640	0.690	2.45	7.92
SMC 880-13B	2:1 DSPC:chol	10mM succinate	membrane	1.980	1.790	3.50	3.24

Table 2B. Comparative Rat Pharmacokinetics Following 1mg/kg IV dose of Free or Liposomal GI147211C

Lot #	Lipid	Counterion	Loading	AUC (µg*hr/ml)	C_{max} (0.25 hr) (µg/ml)	$t_{1/2}$ (hr)	CL (L/kg/hr)
SMC 880-09C	-	-	free drug	0.136	0.100	1.17	7.38
SMC 880-55D	2:1:0.1 DSPC:chol:DSPG	50mM citric acid	passive	19.12	11.45	1.04	0.053
SMC 880-56C	2:1 DSPC:chol	50mM citric acid	passive	15.79	9.78	1.11	0.063
NA908-73	2:1 DSPC:chol	50mM ammonium sulfate	active	106	19.3	8.44	0.013
NA943-050B	2:1 DSPC:chol	50mM citric acid	active	159	22.1	3.2	0.0060
ALM933-030	2:1 HSPC:chol	50mM citric acid	active NH₄Cl quench	133	19.6	4.4	0.0076

Table 3. Liposomal Formulations of GI147211 Loaded at 20:1 Lipid:Drug Ratio. All samples have internally entrapped counterion of 50 mM ammonium citrate, pH 4.5

Molar Ratio	Lipid Formulation	GI147211 (mg/ml)	Median Diameter (nm)	pH	% Drug Loading
2:0.5	DSPC:chol	3.2	77	6.7	63
2:0.75	DSPC:chol	4.2	67	6.8	83
2:1	DSPC:chol	4.4	60	6.9	89
2:1.5	DSPC:chol	4.4	67	6.6	86
1.5:0.5	DSPC:chol	4.2	71	6.6	81
2:1	DPPC:chol	4.3	49	6.0	86
2:1	DMPC:chol	0.9	40	5.9	18
2:1	Egg PC:chol	3.8	61	6.2	79
2:1	HSPC:chol	3.6	62	6.6	73

Table 4. Liposomal Formulations of GI147211 Loaded at Different Drug Lipid Ratios. All samples have internally entrapped counterion of 50 mM ammonium citrate, pH 4.5. Lipid molar ratio is 2:1 PC:cholesterol.

Lipid Formulation	Lipid:Drug Ratio at Loading	GI147211 (mg/ml)	Median Diameter (nm)	PH	% Drug Loading
DSPC:chol	5:1	10.6	63	6.7	53
DSPC:chol	10:1	8.3	65	6.4	85
DSPC:chol	20:1	4.4	60	6.9	89
DSPC:chol	40:1	2.5	60	7.0	91
HSPC:chol	10:1	6.7	62	6.6	61
HSPC:chol	20:1	3.6	62	6.6	73
HSPC:chol	40:1	1.8	63	7.1	70

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Table 5. Percent drug loading of liposomal G1147211 as a function of encapsulated buffer pH. All samples have internally entrapped counterion of 50 mM ammonium citrate or 50 mM ammonium sulfate as indicated. Lipid molar ratio is 2:1 DSPC:cholesterol.

Counterion	Internal pH	% Drug Loading
sulfate	7.0	53
sulfate	6.0	64
sulfate	4.4	77
sulfate	2.1	89
citrate	7.4	70
citrate	6.1	70
citrate	5.8	62
citrate	4.5	81

Table 6. Precipitation of GI147211 Salts

Counterion excipient	Counterion oncentration (mM	% GI147211 Remaining in Solution	% GI147211 Precipitated
sulfate	20	2.1	97.9
sulfate	50	1.9	98.1
citrate	20	1.2	98.8
citrate	50	0.9	99.1
succinate	500	1.2	98.8
chloride	100	14.8	85.2
phosphate	100	17.7	82.3
formate	50	35.3	64.7
aspartate	50	42.2	57.8
sucrose	300	91.8	8.2
lactobionate	50	93.8	6.2

Table 7. Characterization of Liposomal GI147211 Samples for *In Vitro, In Vivo* and Pharmacokinetic Evaluations

Lot Number	Description	GI147211 (mg/ml)	PC (mg/ml)	Cholesterol (mg/ml)	Final Lipid:Drug Ratio	Median Diameter (nm)	pH
SMC880-09C	Free drug	3.0	-	-	-	-	-
SMC880-13B	2:1 DSPC:chol (membrane) 9% sucrose/10mM succinate	5.1	nd	nd	nd	47*	5.4
SMC880-55D	2:1:0.1 DSPC:chol:DSPG (passive loading) 9% sucrose/50 mM citric acid	0.5	31.2	7.6	84:1	57	5.7
SMC880-56C	2:1 DSPC:chol (passive loading) 9% sucrose/50mM citric acid	0.7	50.1	12.3	89:1	60	5.6
SMC880-79	2:1 DSPC:chol 50 mM ammonium sulfate	2.2	40.1	12.7	20:1	67	6.4
SMC880-80	2:1 DSPC:chol 150 mM ammonium phosphate	2.0	41.7	12.9	24:1	67	6.4
SMC880-81	2:1 DSPC:chol 150 mM sodium chloride	0.5	35.9	12.9	104:1	63	5.5
SMC880-082	2:1 DSPC:chol 50 mM citric acid	3.0	44.4	14.6	26:1	57	5.8
SMC880-83	2:1:0.05 DSPC:chol:DSPG 50 mM citric acid	2.5	37.3	12.7	20:1	44	5.6
NA908-073	2:1 DSPC:chol 50 mM ammonium sulfate	2.0	32.3	7.9	20:1	62	6.1

Table 7. (Continued)

NA943-50B	2:1 DSPC:chol 50 mM citric acid	3.4	55.9	14.1	21:1	58	6.0
NA943-72B	2:1 DSPC:chol 50 mM citric acid	3.1	49.7	12.3	20:1	60	5.9
SC974-021-1	2:1 DSPC:chol 50mM citric acid	2.2	37.4	8.5	21:1	60	6.8
ALM993-023-1	2:1 HSPC:chol 100mM citric acid	2.5	41.7	10.5	21:1	70**	4.3
ALM993-023-2	2:1 DSPC:chol 600mM ammonium sulfate 10mM NH$_4$Cl quench	3.4	50.2	12.2	18:1	43	6.5
ALM993-028	2:1 HSPC:chol 50mM citric acid 10mM NH$_4$Cl quench	2.5	37.4	9.5	19:1	65	6.5
ALM993-030	2:1 HSPC:chol 50mM citric acid 10mM NH$_4$Cl quench	2.3	35.6	8.9	20:1	64	6.5

nd   not determined
*   77% volume in main peak
**   89% volume in main peak

Table 8. Median IC$_{50}$ Values (ng/ml)| Range [Minimum| Maximum] and Statistical Comparisons

Cell Line	Tumor Type	liposomal GI147211			GI147211			Topotecan		
		Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum
A549	Lung	8.22E-01	3.10E-01	7.40E+00	6.94E+00	[2.61E+00	1.54E+01	-	-	-
A673	Rhabdomyosarcoma	7.43E-03	9.05E-28	1.05E+01 β	5.80E-01	[6.00E-02	3.74E+00	6.04E+00	2.09E+00	2.16E+01 αδ
B16-Fl	Melanoma (murine)	2.08E+02	6.44E+01	8.35E+02	1.11E+02	[5.98E+00	2.42E+02 β	3.93E+02	4.31E+01	8.95E+02
BT-20	Breast	9.06E-04	1.97E-04	1.52E-01 δ	8.66E-02	[3.33E-02	3.23E-01 α	3.50E+00	-	-
DU-145	Prostate	4.42E-01	1.17E-02	1.06E+01 βδ	1.61E+00	[2.00E-02	9.07E+00 αβ	1.35E+01	5.54E+00	1.75E+01 αδ
ES-2	Ovarian	3.00E-02	6.06E-05	7.17E-01 βδ	4.89E-01	[0.00E+00	2.14E+00 αβ	3.89E+00	3.45E+00	4.06E+02 αδ
H460	Lung, NSC	5.90E-01	3.00E-02	4.60E+00 δ	3.61E+00	[1.50E-01	6.09E+00 α	2.09E+01	2.06E+01	2.11E+01 φ
HT-29	Colon	1.14E-01	4.46E-21	2.23E+00 βδ	7.63E-01	[1.18E-02	3.85E+00 αβ	4.15E+00	6.78E-01	2.76E+01 αδ
KB	Oralpharyngial	3.47E+00	1.15E-02	3.05E+01 β	4.31E+00	[3.13E-01	2.75E+01 β	3.36E+01	1.28E+01	4.19E+01 αδ
KBV	Oralpharyngial ξ	4.53E+02	9.39E+01	1.24E+03	5.84E+02	[2.69E+02	6.66E+04	6.11E+02	5.89E+02	6.70E+02
LOX	Melanoma	5.29E+00	9.82E-02	1.43E+01	1.41E+00	[1.50E+00	5.33E+00	4.92E+00	3.01E+00	6.82E+00 φ
LS-513	Cecum	7.45E-01	6.84E-02	5.83E+00 βδ	4.89E+00	[1.22E+00	2.64E+01 αβ	1.60E+01	1.15E+01	2.35E+01 αδ
MCF-7	Breast	7.14E+01	4.44E+01	1.90E+02 βδ	4.41E+01	[1.93E+01	8.16E+01 αβ	2.30E+02	1.82E+02	4.89E+02 αδ
MDA-MB-468	Breast	2.85E+00	7.33E-01	8.00E+00 β	6.42E+00	[6.40E-01	1.57E+01	4.73E+01	3.57E+00	6.98E+01 α
SKOV-3	Ovarian	8.39E-01	8.37E-02	9.39E+00	1.63E+00	[3.91E-04	1.90E+03	5.18E+00	1.34E-03	7.28E+00
U251	Glioblastoma	3.40E+00	1.48E-01	3.39E-01 β	3.11E+00	[9.00E-02	1.13E+01 β	1.31E+01	2.83E+00	2.22E+01 αδ
U87-MG	Astrocytoma	1.88E-01	1.09E-24	3.07E+00 βδ	2.36E+00	[1.47E-02	1.79E+01 αβ	2.16E+01	1.18E+01	1.89E+02 αδ
U937	Lymphoma	3.78E+00	3.14E-02	1.94E+01 β	4.22E+00	[1.70E-01	1.56E+01 β	9.03E+00	3.49E+00	4.06E+01 αδ

α = Significantly different from liposomal GI147211| p≤0.05   β = Significantly different from topotecan| p≤0.05

δ = Significantly different from GI147211|C| p≤0.05   φ = Insufficient for statistical analysis (n=2 replicate experiments)

ξ = Vincristine Resistant Cell Line   NSC = Non-Small Cell

Table 9.

Drug	Study#	Formulations	Species	No. of animals	Dose (mg/kg)	AUC (total) (µg*hr/mL)	Cmax (µg/mL)	t1/2 (hr)	CL (L/kg/hr)	Vss (L/kg)
SMC-880-09C	P-9707	free	rat	2	1	0.136	0.100	1.17	7.38	9.23
NA-908-73	P-9710	Ammonium sulfate DSPC	rat	3	0.2	16.20	3.09	4.28	0.012	0.056
NA-908-73	P-9710	Ammonium sulfate DSPC	rat	3	1.0	80.54	14.66	8.44	0.013	0.065
NA-908-73	P-9710	Ammonium sulfate DSPC	rat	3	5	472.68	75.94	8.63	0.011	0.067
SMC-880-09C	P-9711	free	mice	18	1	0.14	0.080	1.89	7.30	17.60
NA-908-73	P-9711	Ammonium sulfate DSPC	mice	18	1	64.81	12.140	2.77	0.0154	0.0633
NA-943-50B	P-9719	citrate 0.05 M DSPC	rat	3	1	159.00	22.10	3.15	0.0060	0.030
NA943-072B	980003	citrate 0.05 M DSPC	rat	5	2.5	355.34	48.12	6.10	0.0072	0.041
NA943-072B	980003	citrate 0.05 M DSPC	rat	5	0.5	56.06	8.34	5.54	0.0091	0.048
NA943-072B	980003	citrate 0.05 M DSPC	rat	5	0.1	14.88	2.18	5.13	0.0068	0.036
NA943-072B	980003	citrate 0.05 M DSPC	rat	5	0.02	2.32	0.38	3.03	0.0086	0.042
ALM993-023-1	980072	citrate 0.1 M HSPC	rat	3	1	147	19.8	4.9	0.0068	0.039
ALM993-030	980110	citrate 0.05 M HSPC	rat	5	1	133.06	19.6	4.41	0.0076	0.040

Table 10. Efficacy Comparison of GI147211 and Liposomal GI147211 (NA908-73)**** at Identical Dose.

Tumor	Dose Group	T-C Days	Deaths	Cures	%TGI	Day	Log10 Cell Kill
SW48 Colon Tumor	Empty Liposome	0	0//8	0//8	0	35	
	GI147211 9mg/kg	40	1//8	0//8	86%		1.9
	Liposomal GI147211 6mg/kg	63	0//8	0//8	92%		3
	Liposomal GI147211 9mg/kg	65	0//4	0//8	95%		3.1
KB Head/Neck Tumor	Empty Liposome	0	2//8	0//8	0	22	
	GI147211 6mg/kg	9	0//8	0//8	54%		0.87
	GI147211 9mg/kg	17	0//8	0//8	73%		1.64
	Liposomal GI147211 6mg/kg	43	0//8	0//8	98%		4.14
	Liposomal GI147211 9mg/kg	74	0//8	1//8	99%		7.13
KBV Head/Neck Tumor MDR +	Empty Liposome	0	0//8	0//8	0	18	
	GI147211 9mg/kg	2	0//8	0//8	11%		0.125
	Liposomal GI147211 6mg/kg	14.5	1//8 ***	0//8	54%		0.906
	Liposomal GI147211 9mg/kg	28.5	2//8 **	0//8	65%		1.78
HT29 Colon Tumor	Empty Liposome	0	0//8	0//8	0	26	
	GI147211 6mg/kg	12	0//8	0//8	48%		0.753
	GI147211 9mg/kg	12	0//8	0//8	54%		0.753
	GI147211 12mg/kg	13	0//8	0//8	70%		0.815
	GI147211 14mg/kg	31	0//8	0//8	78%		1.94
	Liposomal GI147211 6mg/kg	25	0//8	0//8	86%		1.57

Table 10. (Continued)

Liposomal GI147211 9mg/kg	38	0//8	0//8	95%	2.38
Liposomal GI147211 12mg/kg*	24	3//8	0//8	92%	1.51
Liposomal GI147211 14mg/kg*	25 a	0//8	0//8	82.50%	1.51

* Liposomal GI147211 groups 12 and 14mg/kg received two total doses of drug.

*** Death due to tumor ulceration

** Deaths occurred on day 6, possibly drug toxicity related

****See Table 7 for formulation

Dose Schedule QD7 X 3; iv.

SW48 Study, First dose given ip.

T-C = time difference between treated and control groups to reach 1,000% Tumor Volume

% Tumor Growth Inhibition(%TGI) = $100(1-Wt/Wc)$; Wt and Wc mean tumor volume of treated and control group

Log 10 Cell Kill = $T-C/3.32 (Td)$; Td = tumor doubling time

a, Starting tumor size was twice that of all other groups

Table 11. Comparison of Therapeutic Index

Drug	KB Tumor (LD50/ED60)	ES2 Tumor (LD50/ED80)
Topotecan	0.5	ND*
GI147211	1	1.1
Liposomal GI147211 (NA943-50B)**	2.9	14.4

* ED 80 value never reached
** See Table 7 for formulation and characterization information

Table 12. Comparison of Antitumor Efficacy of Topotecan, GI147211 and Liposomal GI147211 (NA943-50B)* in The ES2 Tumor Xenograft Model.

Drug and Dose	Maximum Body Weight loss	Toxic Deaths	Cures	T-C	Log Cell Kill
Topotecan, 16mg/kg	16%	3	0	7.4	0.58
GI147211, 14mg/kg	9%	3	1	27	2.14
Liposomal GI147211, 9mg/kg	19%	0	3	51.4	4.08

T-C = Difference in days between treated and control groups to reach 400% tumor volume increase.

Log Cell Kill = T-C/ (3.32) (Td); where Td = Tumor doubling time in days

*See Table 7 for formulation and characterization information

Table 13. Anti-Tumor Efficacy of Liposomal GI147211 (NA943-50B)* vs. GI147211 at Equal Toxic Doses in The MDR +, KBV Xenograft Model

Treatment Group	T-C	Toxic Deaths	Log $_{10}$ Cell Kill	Days to 1000%
Empty Liposome Control	NA	0	0	8.57
GI147211, 12mg/kg	7.58	3	0.53	16.15
GI147211, 14mg/kg	8.43	3	0.59	17
GI147211, 16mg/kg	8.43	1	0.59	17
Liposomal GI147211, 4mg/kg	8.43	2	0.59	17
Liposomal GI147211, 6mg/kg	15.15	0	1.06	23.23
Liposomal GI147211, 9mg/kg	23.23	4	1.63	23.72

*See Table 7 for formulation and characterization information

Table 14. Repeat Dose Anti-Tumor Efficacy of GI147211 vs Liposomal GI147211 (NA943-50B)* KB Tumor Xenograft Model

Drug and Dose	Mean Tumor Volume +/- (SD)	Toxic Deaths	% TGI
Empty Liposome	1655 (618)	0/8	0
GI147211 in D5W, 14mg/kg	275 (67)	0/8	83
GI147211 in empty liposomes, 14mg/kg	278 (61)	0/8	83
Liposomal GI147211 9mg/kg	91 (38)	0/8	95
Liposomal GI147211 6mg/kg	114 (77)	1/8	93

% TGI = 100 (1-Wt/Wc); where Wt is the mean tumor volume of the treated group at day 15 and Wc is the mean tumor volume of the control group at day 15.

*See Table 7 for formulation and characterization information.

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Table 15. Comparison of HSPC (ALM 993-028) vs. DSPC (SC 974-021-1) Liposomal
GI147211 Formulations and GI147211 in the KB Xenograft Model.

Drug/Dose	T-C	Toxic Deaths	Cures	% TGI	Log Cell Kill Index
D5W control	0	0/8	0/8	0%	0
GI147211, 14mg/kg	13.4	0/8	0/8	77.5%	0.81
Liposomal GI147211					
SC974-021-1; 3mg/kg	40.2	0/8	1/8	92.9%	2.42
SC974-021-1; 6mg/kg	45.8	0/8	2/8	96.2%	2.75
SC974-021-1; 9mg/kg	73	0/8	1/8	98.1%	4.39
ALM993-028; 3mg/kg	35	0/8	0/8	91%	2.37
ALM993-028; 6mg/kg	45.8	0/8	1/8	94.6%	2.75
ALM993-028; 9mg/kg	53.4	1/8	1/8	96.6%	3.21

T-C = Time difference between treated and control groups to achieve 400% tumor volume
increase.
% Tumor Growth Inhibition (%TGI) = 100(1-Wt/Wc); Wt and Wc represent mean tumor
volume of treated and control groups at day 18.
Log cell Kill Index = T-C/3.32(Td); Td = Tumor doubling time.

Cure refers to tumor-free animals at day 60.

Table 16.

Routes of Administration in Mice

Drug	Study#	Formulations	Species	No. of animals	Drug	Dose (mg/kg)	Dosing route	AUC (total) (µg*hr/mL)	Cmax (µg/mL)	Tmax (hr)	CL (L/kg/hr)	Vss (L/kg)
SMC-880-09C	P-9711		mice	18	free	1	I.V.	0.14	0.080	0.00	7.3038	17.6047
NA-908-73		Ammonium sulfate	mice	18	liposome	1	I.V.	64.81	12.140	0.00	0.0154	0.0633
SMC-880-09C			mice	18	free	1	I.P.	0.047	0.056	0.25	N/A	N/A
NA-908-73		Ammonium sulfate	mice	18	liposome	1	I.P.	23.43	3.558	4.00	N/A	N/A
SMC-880-09C			mice	18	free	1	Sub. Q	0.055	0.042	0.25	N/A	N/A
NA-908-73		Ammonium sulfate	mice	18	liposome	1	Sub. Q	10.569	1.349	4.00	N/A	N/A

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We claim:

1.   A liposome comprising at least one phospholipid and 7-(4-methylpiperazinomethylene)-10,11-ethylenedioxy-20(S)-camptothecin (GI147211).

5   2.   The liposome of claim 1 wherein said at least one phospholipid is a phosphatidylcholine.

3.   The liposome of claim 2 wherein said phopshatidylcholine is selected from the group consisting of distearoylphosphatidylcholine, hydrogenated soy

10   phosphatidylcholine, soy phosphatidylcholine, egg phosphatidylcholine, hydrogenated egg phosphatidylcholine, dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine.

4.   The liposome of claim 3 wherein said phosphatidylcholine is hydrogenated soy phosphatidylcholine.

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5.   The liposome of claim 2 wherein said liposome further comprises cholesterol.

6.   The liposome of claim 4 wherein said liposome further comprises cholesterol.

20   7.   The liposome of claim 2 wherein said liposome further comprises phosphatidylglycerol.

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8.   The liposome of claim 7 wherein said phosphatidylglycerol is selected from the group consisting of dimyristoylphosphatidylglycerol, dilaurylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, distearoylphosphatidylglycerol.

5   9.   The liposome of claim 8 wherein said phosphatidylglycerol is distearoylphosphatidylglycerol.

10.   The liposome of claim 6 wherein said liposome further comprises phosphatidylglycerol.

10

11.   The liposome of claim 10 wherein said phosphatidylglycerol is selected from the group consisting of dimyristoylphosphatidylglycerol, dilaurylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, distearoylphosphatidylglycerol.

15   12.   The liposome of claim 11 wherein said phosphatidylglycerol is distearoylphosphatidylglycerol.

13.   The liposome of claim 1 wherein a counterion is used to initiate or facilitate the loading of said GI147211 into said liposome.

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14.   The liposome of claim 13 wherein said counterion is an acid, a sodium or an ammonium form of a monovalent anion, a divalent anion or trivalent anion.

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15.   The liposome of claim 14 wherein said monovalent anion is chloride, acetate, lactobionate or formate; said divalent anion is aspartate, succinate or sulfate; and said trivalent anion is citrate or phosphate.

5   16.   The liposome of claim 5 wherein the phosphatidylcholine to cholesterol molar ratio is from about 1.5:0.5 to 2:1.5.

17.   The liposome of claim 16 wherein said molar ratio is about 2:1.

10   18.   The liposome of claim 17 wherein said liposome is unilamellar and less than 100 nm.

19.   The liposome of claim 18 wherein said lipid to GI147211 molar ratio is from about 5:1 to 100:1.

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20.   The liposome of claim 19 wherein said lipid to GI147211 molar ratio is from about 5:1 to 40:1.

21.   The liposome of claim 20 wherein said lipid to GI147211 molar ratio is about 20   20:1.

22.   The liposome of claim 6 wherein the hydrogenated soy phosphatidylcholine to cholesterol molar ratio is from about 1.5:0.5 to 2:1.5.

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23.   The liposome of claim 22 wherein said molar ratio is about 2:1.

24.   The liposome of claim 23 wherein said liposome is unilamellar and less than 100 nm.

5

25.   The liposome of claim 24 wherein said lipid to GI147211 molar ratio is from about 5:1 to 100:1.

26.   The liposome of claim 25 wherein said lipid to GI147211 molar ratio is from 10 about 5:1 to 40:1.

27.   The liposome of claim 26 wherein said lipid to GI147211 molar ratio is 20:1.

28.   The liposome of claim 27 wherein a counterion is used to used to initiate or 15 facilitate the loading of said GI147211 into said liposome.

29.   The liposome of claim 28 wherein said counterion is an acid, a sodium or an ammonium form of a monovalent anion, a divalent anion or trivalent anion.

20   30.   The liposome of claim 29 wherein said monovalent anion is chloride, acetate, lactobionate or formate; said divalent anion is aspartate, succinate or sulfate; and said trivalent anion is citrate or phosphate.

31.   The liposome of claim 30 wherein said counterion is citrate.

32. The composition of claim 5 produced by the process comprising:

a) forming a lipid film or powder comprised of phosphatidylcholine and cholesterol;

5 b) hydrating said lipid film or powder with an aqueous buffer containing a counterion solution;

c) applying a shearing force whereby liposomes that are unilamellar and less than 100 nm are obtained;

d) adding GI147211 to the aqueous medium; and

10 e) generating a gradient between the aqueous medium inside the liposome and the aqueous medium external to the liposome whereby the gradient causes the GI147211 to load into the liposome.

33. The method of claim 32 wherein the gradient is a pH gradient.

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34. The method of claim 32 wherein the phosphatidylcholine to cholesterol molar ratio is from about 1.5:0.5 to 2:1.5.

35. A method of inhibiting the growth of a tumor comprising the administration

20 of a therapeutic or effective amount of the composition of claim 1 to a tumor.

36. The method of claim 35 wherein said tumor is drug resistant or drug sensitive.

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37.   The method of claim 36 wherein said tumor is from a cancer selected from the group consisting of ovarian, small cell lung cancer, non-small cell lung cancer, colorectal cancer, breast cancer, and head and neck cancer.

5   38.   A liposome comprising a camptothecin encapsulated in a liposome, wherein said liposome is comprised of hydrogenated soy phosphatidylcholine (HSPC) and cholesterol and wherein HSPC:cholesterol are in a molar ratio of about 2:1, and wherein the lipid:camptothecin molar ratio is from 10:1 to 40:1, and wherein said liposomes are unilamellar having an average size of less than 100 nm.

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39.   The composition of claim 38 produced by the process comprising

a)   forming a lipid film or powder comprised of phosphatidylcholine and cholesterol;

b)   hydrating said lipid film or powder with an aqueous buffer containing a

15   counterion solution;

c)   applying a shearing force whereby liposomes that are unilamellar and less than 100 nm are obtained;

d)   adding camptothecin to the aqeuous medium; and

e)   generating a gradient between the aqueous medium inside the liposome and

20   the aqueous medium external to the liposome whereby the gradient causes the camptothecin to load into the liposome.

40.   The method of claim 39 wherein the gradient is a pH gradient.

53

41.   The method of claim 39 wherein the phosphatidylcholine to cholesterol molar ratio is from about 1.5:0.5 to 2:1.5.

42.   A method of inhibiting the growth of a tumor comprising the administration

5   of a therapeutic or effective amount of the composition of claim 38 to a tumor.

43.   The method of claim 42 wherein said tumor is drug resistant or drug sensitive.

10   44.   The method of claim 43 wherein said tumor is from a cancer selected from the group consisting of ovarian, small cell lung cancer, non-small cell lung cancer, colorectal cancer, breast cancer, and head and neck cancer.

45.   A process for making liposomes comprising GI147211, said method

15   comprising:

a)   forming a lipid film or powder comprised of phosphatidylcholine and cholesterol;

b)   hydrating said lipid film or powder with an aqueous buffer containing a counterion solution;

20   c)   applying a shearing force whereby liposomes that are unilamellar and less than 100 nm are obtained;

d)   adding GI147211 to the aqueous medium; and

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e)   generating a gradient between the aqueous medium inside the liposome and
the aqueous medium external to the liposome whereby the gradient causes the GI147211 to
load into the liposome.

5   46.   The method of claim 45 wherein the phosphatidylcholine to cholesterol molar
ratio is from about 1.5:0.5 to 2:1.5.

47.   The method of claim 45 wherein the gradient is a pH gradient.

10   48.   The method of claim 47 wherein the pH of the aqueous medium inside the
liposome relative to the aqueous medium external to the liposome is less.

49.   The method of claim 48 further comprising increasing the pH of the aqueous
medium inside of the liposome by adding a membrane permeable amine to the aqueous
15   medium external to the liposome.

50.   The method of claim 49 wherein said membrane permeable amine is selected
from the group consisting of an ammonium salt and an alkyl-amine.

20   51.   The method of claim 50 wherein said ammonium salt has a mono- or multi-
valent counterion.

52.   The method of claim 51 wherein said ammonium salt is selected from the
group consisting of ammonium sulfate, ammonium hydroxide, ammonium acetate,

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ammonium chloride, ammonium phosphate, ammonium citrate, ammonium succinate,

ammonium lactobionate, ammonium carbonate, ammonium tartrate, and ammonium oxalate.

53.   The method of claim 52 wherein said alkyl-amine is selected from the group

5   consisting of methylamine, ethylamine, diethylamine, ethylenediamine and propylamine.

54.   The method of claim 45 wherein said phosphatidylcholine is selected from

the group consisting of distearoylphosphatidylcholine, hydrogenated soy

phosphatidylcholine, soy phosphatidylcholine, egg phosphatidylcholine, hydrogenated egg

10   phosphatidylcholine, dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine.

55.   The method of claim 54 wherein said phosphatidylcholine is hydrogenated

soy phosphatidylcholine.

15   56.   The method of claim 55 wherein said liposome further comprises a

phosphatidylglycerol.

57.   The method of claim 56 wherein said phosphatidylglycerol is selected from

the group consisting of dimyristoylphosphatidylglycerol, dilaurylphosphatidylglycerol,

20   dipalmitoylphosphatidylglycerol, distearoylphosphatidylglycerol.

58.   The method of claim 57 wherein said phosphatidylglycerol is

distearoylphosphatidylglycerol.

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59.   The method of claim 45 wherein said phosphatidylcholine to cholesterol molar ratio is about 2:1.

60.   The method of claim 45 wherein said lipid to GI147211 molar ratio is from about 10:1 to 40:1.

61.   The method of claim 60 wherein said lipid to GI147211 molar ratio is 20:1.

62.   A process for making liposomes comprising camptothecin, said method comprising:

    a)   forming a lipid film or powder comprised of phosphatidylcholine and cholesterol;

    b)   hydrating said lipid film or powder with an aqueous buffer containing a counterion solution;

    c)   applying a shearing force whereby liposomes that are unilamellar and less than 100 nm are obtained;

    d)   adding camptothecin to the aqueous buffer; and

    e)   generating a gradient between the aqueous medium inside the liposome and the aqueous medium external to the liposome whereby the gradient causes the camptothecin to load into the liposome.

63.   The method of claim 62 wherein the phosphatidylcholine to cholesterol molar ratio is from about 1.5:0.5 to 2:1.5.

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64.   The method of claim 62 wherein the gradient is a pH gradient.

65.   The method of claim 64 wherein the pH of the aqueous medium inside the

liposome relative to the aqueous medium external to the liposome is less.

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66.   The method of claim 65 further comprising increasing the pH of the aqueous

medium inside of the liposome by adding a membrane permeable amine to the aqueous

medium external to the liposome.

10   67.   The method of claim 66 wherein wherein said membrane permeable amine is

selected from the group consisting of an ammonium salt and an alkyl-amine.

68.   The method of claim 66 wherein said ammonium salt has a mono- or multi-

valent counterion.

15

69.   The method of claim 68 wherein said ammonium salt is selected from the

group consisting of ammonium sulfate, ammonium hydroxide, ammonium acetate,

ammonium chloride, ammonium phosphate, ammonium citrate, ammonium succinate,

ammonium lactobionate, ammonium carbonate, ammonium tartrate, and ammonium oxalate.

20

70.   The method of claim 67 wherein said alkyl-amine is selected from the group

consisting of methylamine, ethylamine, diethylamine, ethylenediamine and propylamine.

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71.   The method of claim 62 wherein said phosphatidylcholine is selected from

the group consisting of distearoylphosphatidylcholine, hydrogenated soy

phosphatidylcholine, soy phosphatidylcholine, egg phosphatidylcholine, hydrogenated egg

phosphatidylcholine, dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine.

5

72.   The method of claim 71 wherein said phosphatidylcholine is hydrogenated

soy phosphatidylcholine.

73.   The method of claim 72 wherein said liposome further comprises a

10   phosphatidylglycerol.

74.   The method of claim 73 wherein said phosphatidylglycerol is selected from

the group consisting of dimyristoylphosphatidylglycerol, dilaurylphosphatidylglycerol,

dipalmitoylphosphatidylglycerol, distearoylphosphatidylglycerol.

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75.   The method of claim 74 wherein said phosphatidylglycerol is

distearoylphosphatidylglycerol.

76.   The method of claim 62 wherein said phosphatidylcholine to cholesterol

20   molar ratio is about 2:1.

77.   The method of claim 62 wherein said lipid to camptothecin molar ratio is

from about 10:1 to 40:1.

78.   The method of claim 77 wherein said lipid to camptothecin molar ratio is

20:1.

79.   A process for making liposomes comprising camptothecin, said method

comprising:

        a)   forming a lipid film or powder comprised of phospholipid and cholesterol;

        b)   hydrating said lipid film or powder with an aqueous buffer containing a

counterion solution;

        c)   adding camptothecin to the aqueous buffer;

        d)   generating a gradient between the aqueous medium inside the liposome and

the aqueous medium external to the liposome whereby the gradient causes the camptothecin

to load into said liposome; and

        e)   adding a membrane permeable amine to the aqueous medium external to the

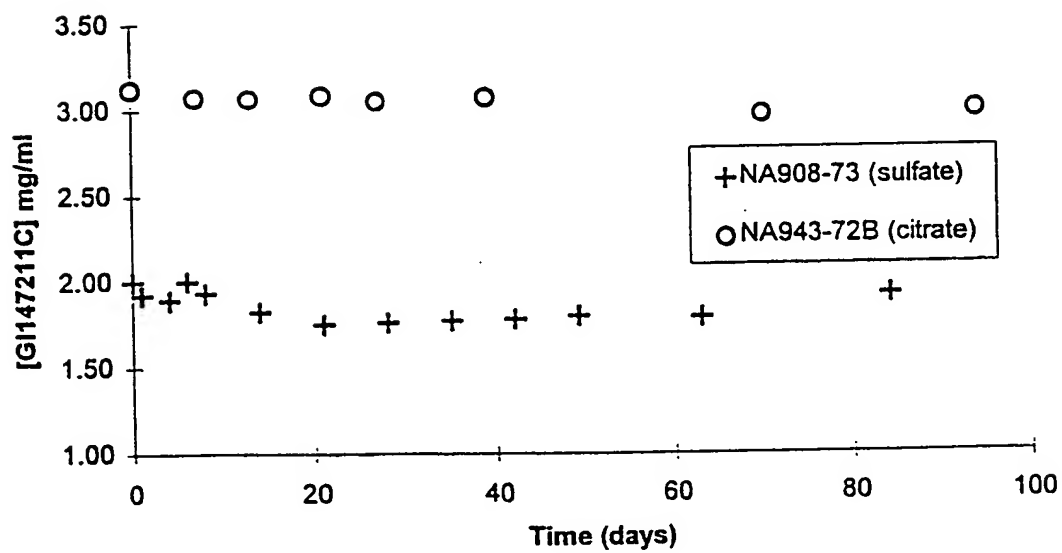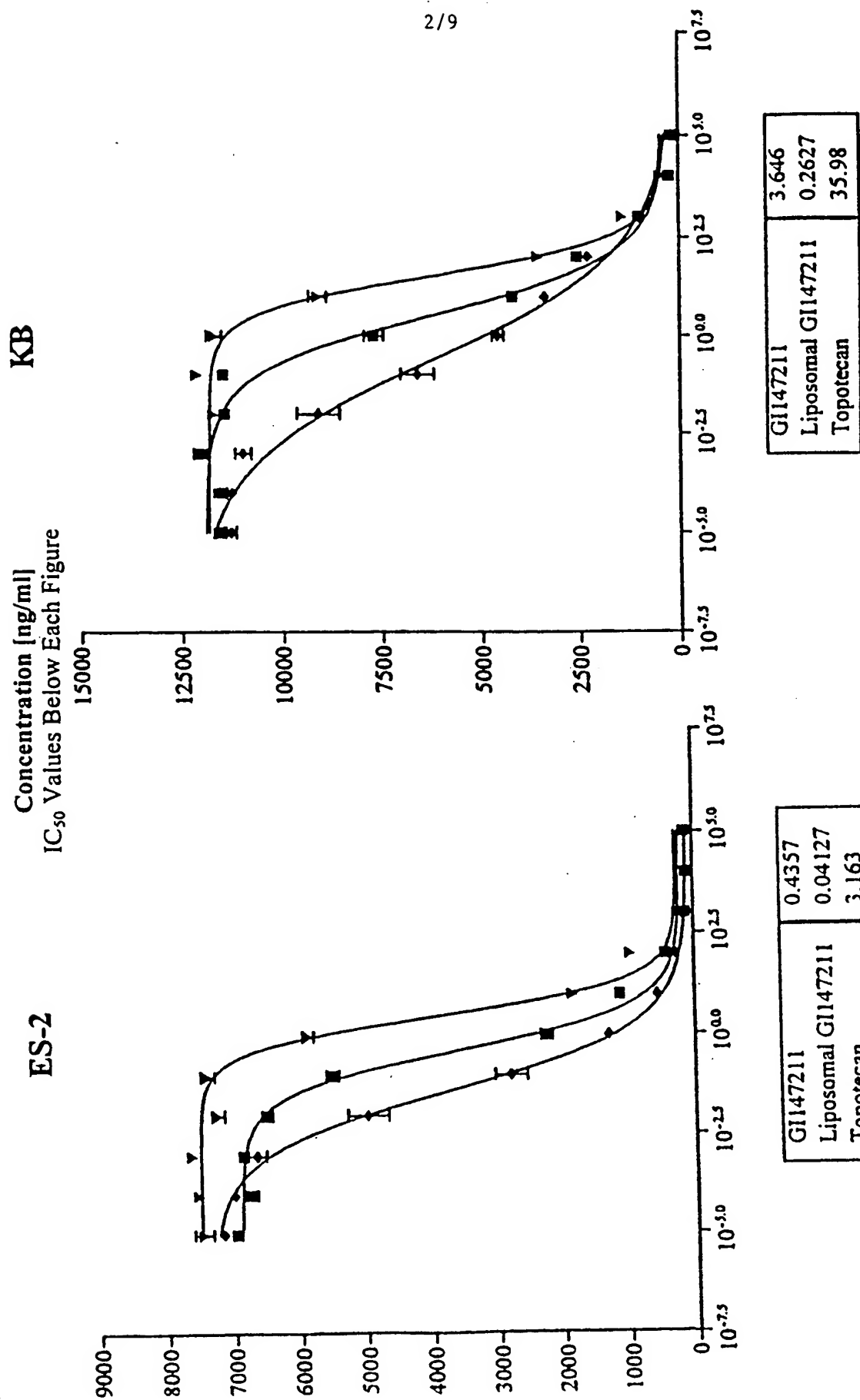liposome whereby the pH of the aqueous medium inside the liposome is decreased.

80.   A liposome comprising a camptothecin encapsulated in a liposome, wherein

said liposome is produced by the method comprising:

        a)   forming a lipid film or powder comprised of phospholipid and cholesterol;

        b)   hydrating said lipid film or powder with an aqueous buffer containing a

counterion solution;

        c)   adding camptothecin to the aqueous buffer;

        d)   generating a gradient between the aqueous medium inside the liposome and

the aqueous medium external to the liposome whereby the gradient causes the camptothecin

to load into said liposome; and

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e)   adding a membrane permeable amine to the aqueous medium external to the

liposome whereby the pH of the aqueous medium inside the liposome is decreased.
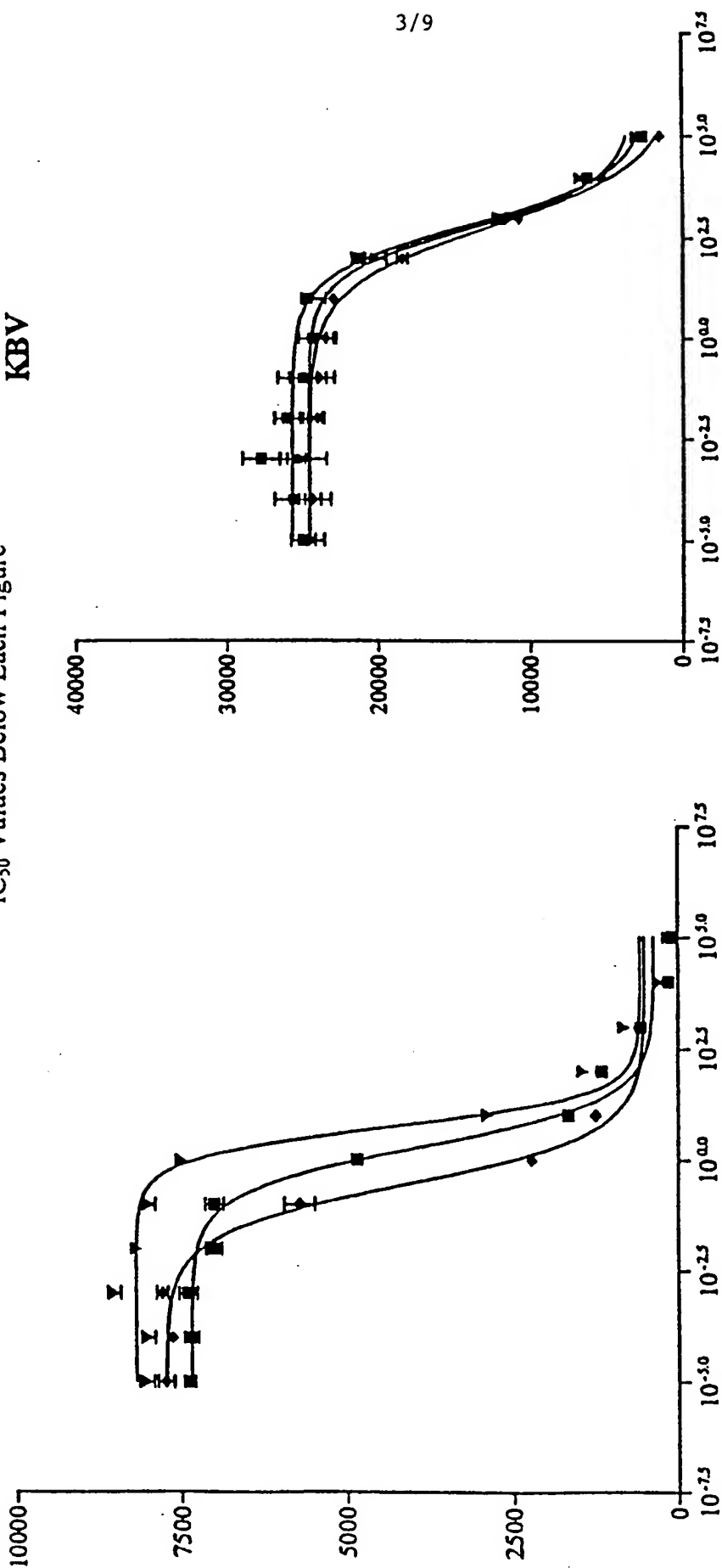
Fig. 1

Fig. 2

ES-2

KB

Concentration [ng/ml]
IC50 Values Below Each Figure

GI147211	3.646
Liposomal GI147211	0.2627
Topotecan	35.98

Fig. 3B

GI147211	0.4357
Liposomal GI147211	0.04127
Topotecan	3.163

Fig. 3A

Concentration [ng/ml]
IC$_{50}$ Values Below Each Figure

KBV

GI147211	719.6
Liposomal GI147211	683.5
Topotecan	601.5

Fig. 3D

SKOV-3

GI147211	2.020
Liposomal GI147211	0.2841
Topotecan	5.431

Fig. 3C

Concentration [ng/ml]
IC$_{50}$ Values Below Each Figure

DU-145
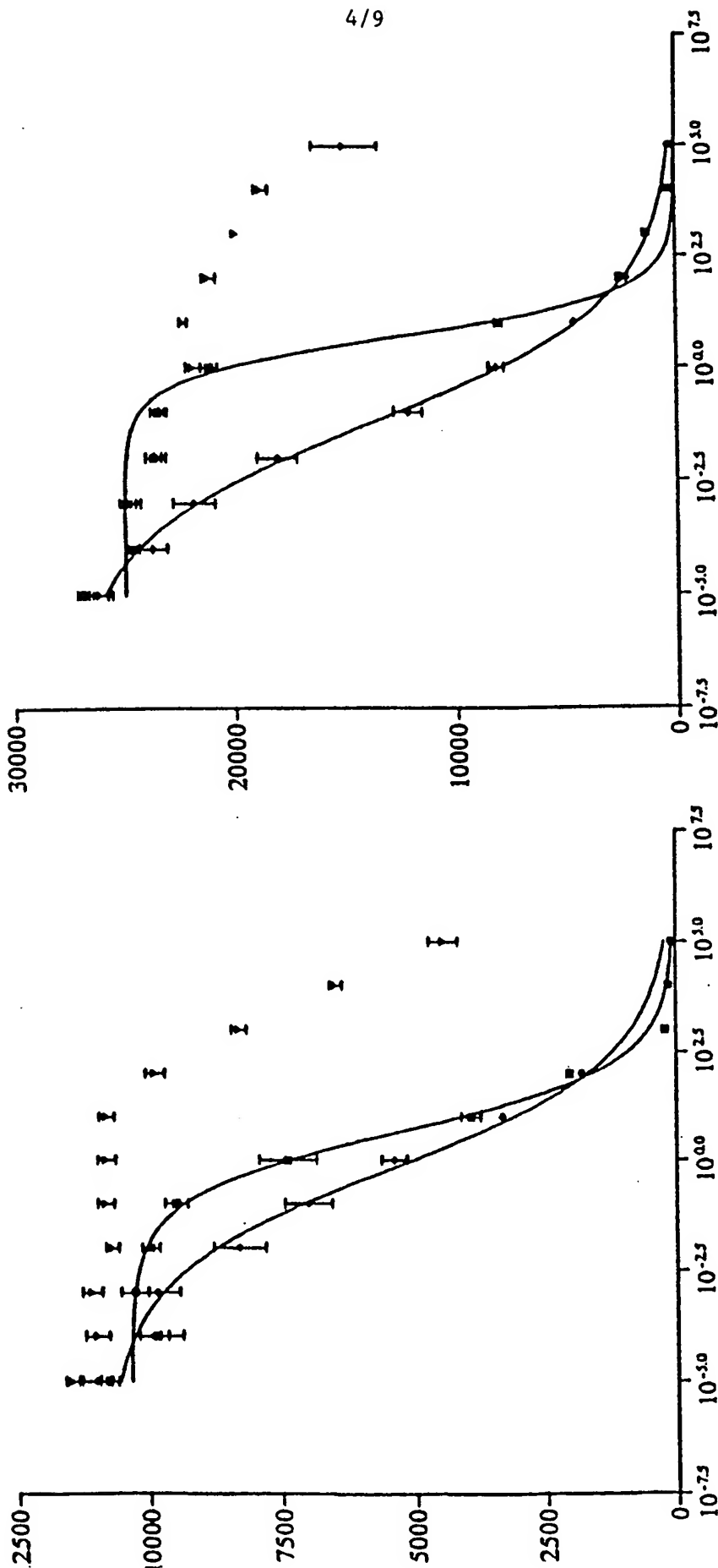
| GI147211 | 5.022 | 0.06047 |
| Liposomal GI147211 | | |

Fig. 4B

A549

| GI147211 | 4.791 | 0.6493 |
| Liposomal GI147211 | | |

Fig. 4A

Fig. 4D

Fig. 4C

Fig. 5

Fig. 6

Fig. 7

Lyso PC (mole%)

$y = 1.2554x + 4.1312$
$R^2 = 0.9436$

$y = -0.0075x + 2.228$
$R^2 = 0.1274$

■ 100mM
● 100mM/NH4Cl

time (weeks)

Fig. 8